STUDIES ON PRODUCTION OF SOME USEFUL SUBSTANCES FROM HYDROCARBONS BY MICROORGANISMS

1968

STUDIES ON PRODUCTION OF SOME USEFUL SUBSTANCES FROM HYDROCARBONS BY MICROORGANISMS

1968

Preface

In the present thesis, the author's studies are collected, which have been carried out under the guidances of Professor Saburo Fukui in his laboratory at Kyoto University during 1965-1968.

The studies contain the investigations on the utilization of petroleum hydrocarbons and the production of the useful materials from hydrocarbons by various microorganisms, such as yeasts, molds and bacteria.

It is a pleasure to express greatful acknowledgements to Professor Saburo Fukui for his continuous direction and encouragement throughout this work.

The author is particularly indebted to Dr. Nobuko Ohishi and to Messrs. Katsuhiko Fujii, Tomohisa Nagasaki, Shin Shimizu, Hidehiko Maki and Masanobu Inagawa for their valuable collaborations and discussions during the course of the studies.

Atsuo Tanaka

Laboratory of Industrial Biochemistry

Department of Industrial Chemistry

Faculty of Engineering

Kyoto University

September, 1968

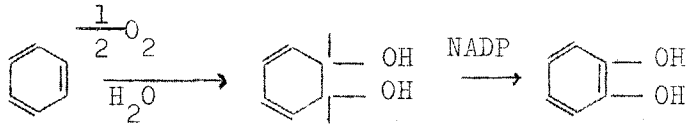
Contents

General Introduction

Since 1895 when Miyoshi[1] found out that a common industrial fungus, Botrytis cinerea, attacked hydrocarbons, many investigations were carried out on hydrocarbon utilization by various microorganisms. In these studies, it has been made clear that a large number of microorganisms, including bacteria, yeasts, and molds, could assimilate aliphatic and/or aromatic hydrocarbons as the sole source of carbon during their propagation, and that the former seemed to be the superior growth substrate for microbes[2,3]. At the present time, most investigators working on petroleum microbiology became to believe that oxidation and assimilation of hydrocarbons were the widespread property of microorganisms.

At present, the oxidation pathways of aromatic and aliphatic hydrocarbons are considered to be as follows: in 1950, Hayaishi et al.[4] found out an enzyme, di-oxygenase, which converted catechol into cis-cis-muconic acid under the presence of molecular oxygen. Independently, Mason[5] proposed an enzyme, mono-oxygenase or mixed-function oxidase, which would participate with the hydroxylation of hydrocarbons. Along with these studies, Evans et al.[6], Stanier et al.[7], and other workers[8] investigated on the metabolic pathways of

aromatic compounds by microorganisms. Most aromatic hydrocarbons will be converted into catechol or protocatechuic acid under the participation of mono-oxygenase and other enzymes, and catechol and its derivatives will be sustained the ring fission by di-oxygenase. The typical metabolic pathways of the aromatic hydrocarbons are shown in Fig. 1, 2 and 3.



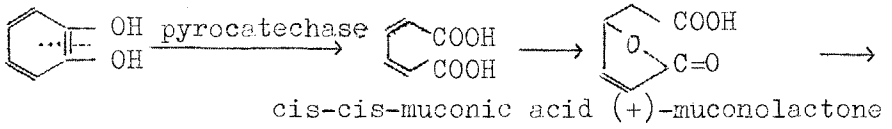
benzene 3,5-cyclohexadiene-1,2-diol catechol

benzene phenol

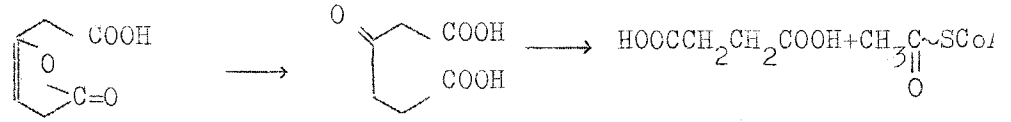
H_2X: Electron donor

Fig. 1. Hydroxylation of benzene ring

a). 1,2-Fission

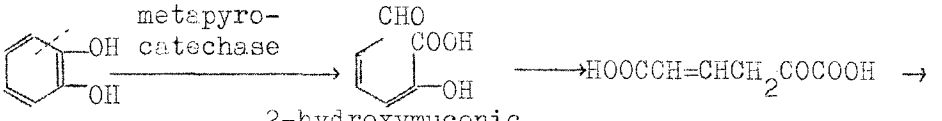


cis-cis-muconic acid (+)-muconolactone

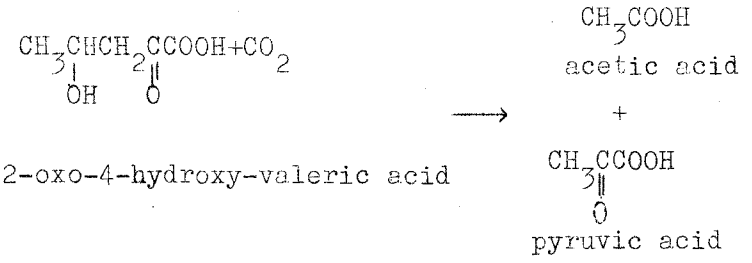


3-oxoadipate enol-lactone 3-oxoadipic acid succinic acid acetyl-CoA

b). 2,3-Fission



2-hydroxymuconic semialdehyde 4-oxalocrotonic acid



2-oxo-4-hydroxy-valeric acid

Fig. 2. Metabolic pathway of catechol

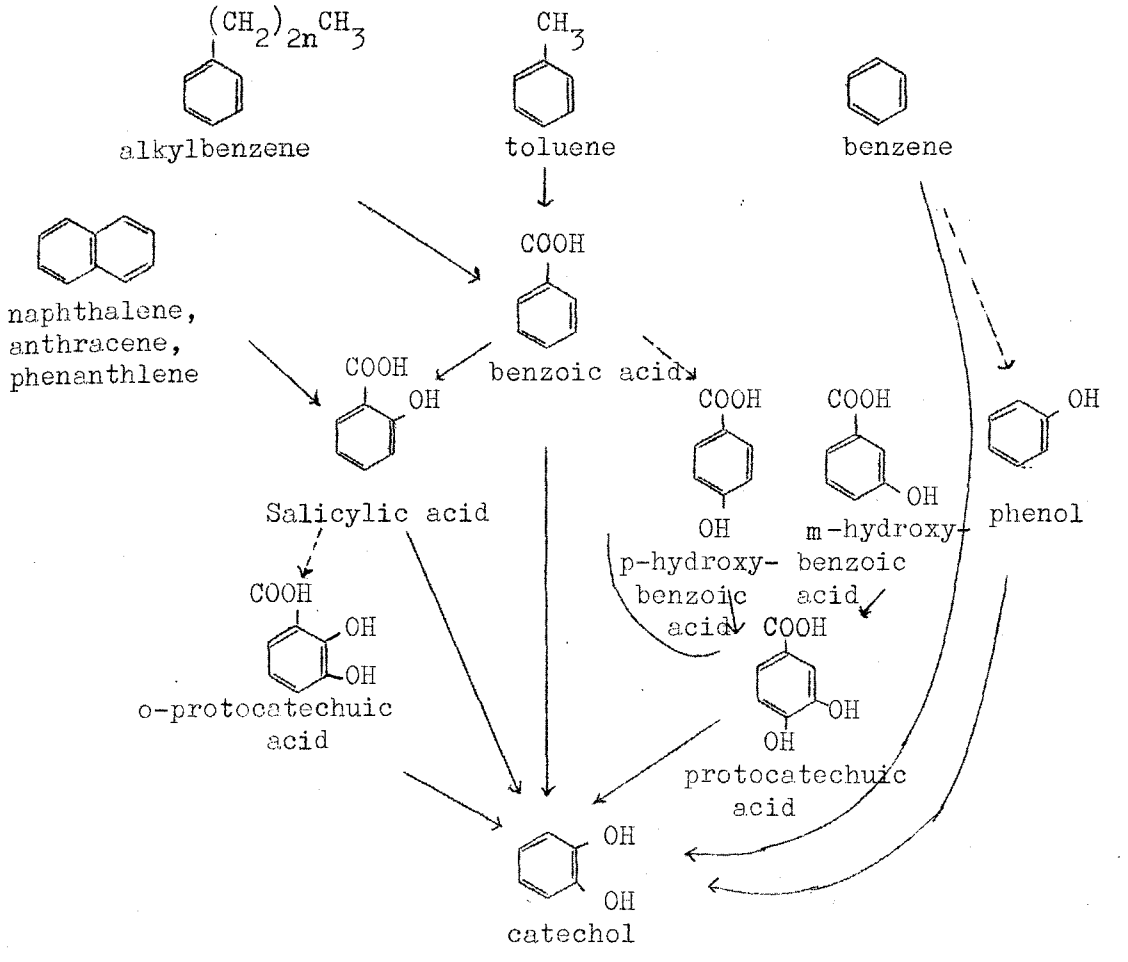


Fig. 3. Formation of catechol and protocatechuic acid from aromatic hydrocarbons

On the other hand, aliphatic hydrocarbons has been believed to be metabolized through aliphatic alcohols and acids. In this case, three mechanisms for alcohol formation have been proposed; that is, (1) formation of alkane peroxide and its reduction[9], (2) participation of mono-oxygenase (mixed-function oxidase)[5], and (3) participation of dehydrogenase and formation of 1-alkenes[10, 11]; but an enzymatic demonstration has been made only about the second mechanism by Kusunose et al[12] and Coon et al[13]. The aliphatic acids thus formed will be metabolized by the well-known β- and ω-oxidation pathways as shown in Fig. 5. Fig. 4 shows the mechanism for the initial oxidation of n-alkanes by mono-oxygenase-dependent system proposed by Kusunose et al[12] and Coon et al[13].

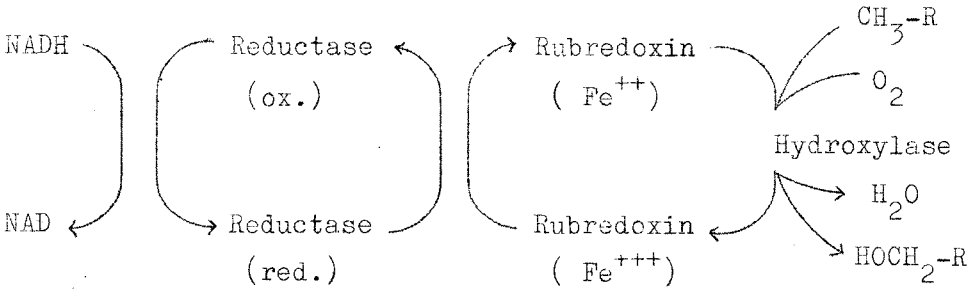


Fig. 4. Hydroxylation of terminal methyl group

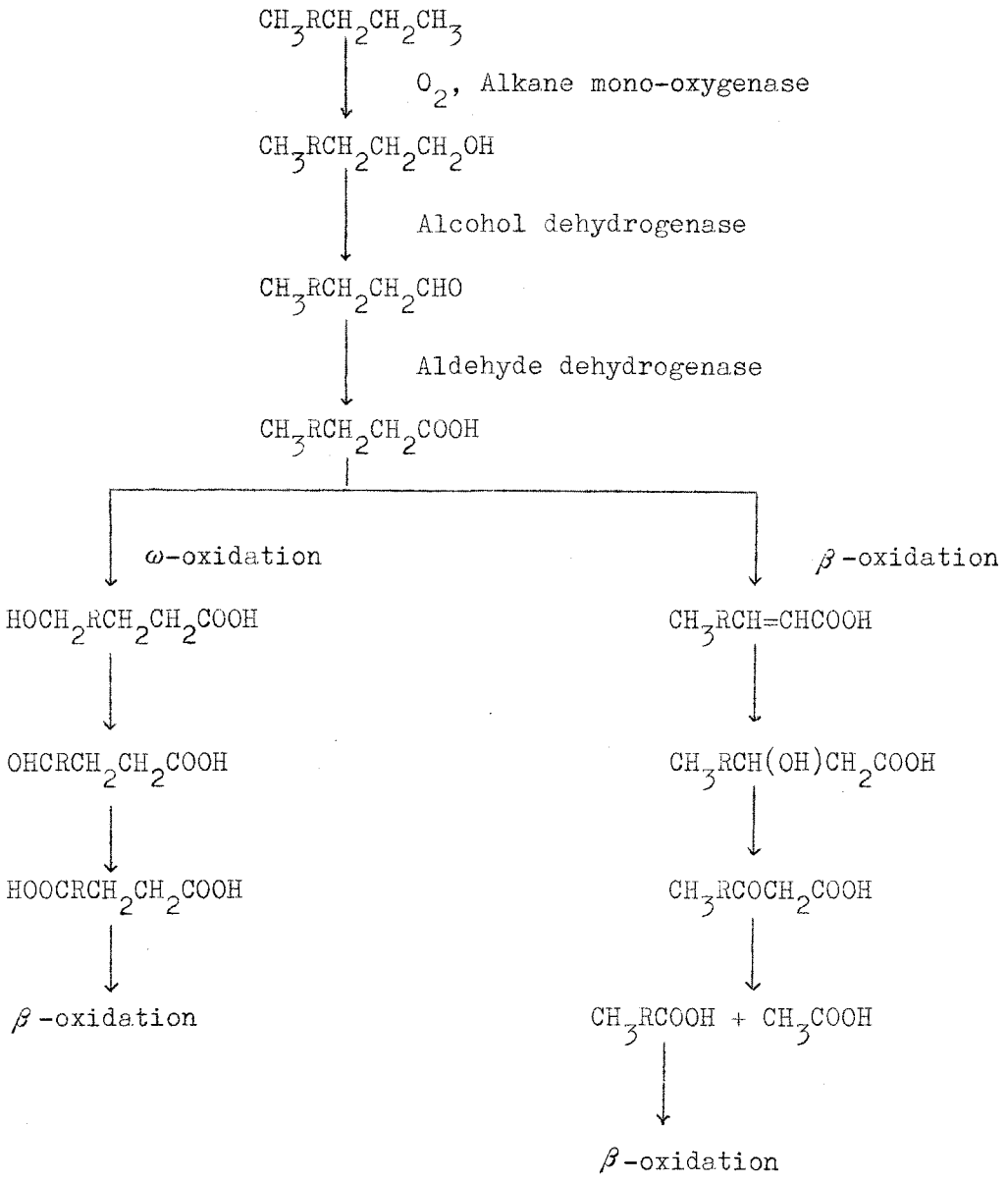


Fig. 5. Metabolic pathway of n-alkanes

Acetic acid or other acids formed from the longer aliphatic acids by the degradative oxidation will be used as substrates for construction of cellular materials via the complex biosynthetic routes.

Besides physiological and biochemical studies on the assimilation of hydrocarbons, the production of useful materials from hydrocarbon substrates has been intensely investigated using the fermentation techniques. These workers have claimed the merit of hydrocarbon fermentation that it would be of deep significance for the human living to convert nonedible materials, hydrocarbons, into the eatable ones by the action of microorganisms.

Thus, a large number of the investigations have been attempted in laboratory and industrial scales in order to produce the yeast cell mass[14, 15], proteins[16], amino acids[17, 18, 19], nucleic acids[20, 21], fatty substances[22, 23], and other useful materials.

The present author has been studied on the formation of vitamins, especially their coenzyme forms, from aliphatic hydrocarbons using a number of microorganisms, including bacteria, yeasts, and molds. Besides vitamin B_6[24] and B_{12}[25], and their coenzyme forms, the production of carotenoid pigments[26], respiratory enzyme (cytochrome c)[27], and porphyrins[28] has

been also studied.

The object of this paper is to study on the hydrocarbon fermentation with the view to produce vitamins and other useful materials for medical use or food supplement.

Part I describes the formation of B_6-vitamers from n-hexadecane by various microorganism, especially yeast Candida albicans.

The factors affecting the growth and cytochrome c production of Candida albicans are dealt in Part II accompanied with the cytochrome c productivity of several kinds of yeast.

In Part III, the formation of carotenoids by Mycobacterium smegmatis and their characteristics are mentioned. The results show that the carotenoid pigment produced by this bacterium mainly consist of 4-keto-γ-carotene and its derivatives.

The characterization of metal-free porphyrins accumulated in the cells and excreted into the culture filtrate by Mycobacterium smegmatis is described in Part IV.

In Part V, the substrate specificity of some yeasts during n-alkane assimilation is investigated, and the growth ability of various yeasts and molds on aromatic and aliphatic hydrocarbons and their derivatives are reported in Part VI.

References

1). M. Miyoshi: Jahrb. Wiss. Botan., 28, 269 (1895)

2). C.E. Zobell: Adv. Enzymol., 10, 443 (1950)(Interscience Publishers, Inc., New York)

3). J.B. Davis: Petroleum Microbiology (1967) (Elsivier Publishing Co., Amsterdam)

4). O. Hayaishi and K. Hashimoto: J. Biochem., 37, 371 (1950)
O. Hayaishi and R.Y. Stanier: J. Bacteriol., 62, 691 (1951)

5). H.S. Mason: Adv. Enzymol., 19, 79 (1957) (Interscience Publishers, Inc., New York)

6). W.C. Evans and B.S.W. Smith: Biochem. J., 49, X (1951)
N. Walker and W.C. Evans: Biochem. J., 52, XXIII (1952)
J.I. Davies and W.C. Evans: Biochem. J., 91, 251 (1964)

7). W.R. Sistrom and R.Y. Stanier: J. Biol. Chem., 210, 821 (1954)
W.R. Sistrom and R.Y. Stanier: Nature, 174, 513 (1954)
L.N. Ornston and R.Y. Stanier: Nature, 204, 1279 (1964)

8). S. Dagley and D.A. Stopher: Biochem. J., 73, 16P (1959)
S. Dagley and D.T. Gibson: J. Biol. Chem., 239, 1284 (1964)
L.N. Ornston: J. Biol. Chem., 241, 3787, 3795, 3800 (1966)

9). B. Imelik: Compt. Rend. Acad. Sci., 227, 1178 (1948)
J.E. Stewart and R.E. Kallio: J. Bacteriol., 78, 726 (1959)

10). H. Iizuka, M. Iida, A. Unami and Y. Hoshino: Seikagaku, 39, 530 (1967)

11). E. Azoulay and J.C. Senez: Compt. Rend. Acad. Sci., 247, 1251 (1958)
J.C. Senez and E. Azoulay: Biochim. Biophys. Acta, 47, 307 (1961)
E. Azoulay and M.T. Heydeman: Biochim. Biophys. Acta, 73, 1 (1963)

12). M. Kusunose, J. Matsumoto, K. Ichihara, E. Kusunose and J. Nozaka: J. Biochem. (Tokyo), 61, 665 (1967)

13). J.A. Peterson, M. Kusunose, E. Kusunose and M.J. Coon: J. Biol. Chem., 242, 4334 (1967)
J.A. Peterson and M.J. Coon: J. Biol. Chem., 243, 329 (1968)

14). A. Champagnat, C.Vernet, B. Laine and J. Filosa: Nature, 197, 13 (1963)

15). T.L. Miller and M.J. Johnson: Biotech. Bioeng., 8, 549, 567 (1966)

16). J. Takahashi, K. Kobayashi, Y. Kawabata and K. Yamada: Agr. Biol. Chem., 27, 836 (1963)

17). K. Yamada, J. Takahashi and K. Kobayashi: Agr. Biol. Chem., 27, 773 (1963)

J. Takahashi, K. Kobayashi, Y. Imada and K. Yamada: Appl. Microbiol., 13, 1 (1965)

Y. Imada, E.C. Pantskhava and K. Yamada: Amino acid. Nucleic acid, 16, 56 (1967)

18). T. Iguchi, I. Takeda and S. Senoh: Agr. Biol. Chem., 29, 589 (1965)

19). R. Uchino, S. Otsuka and I. Shiio: J. Gen. Appl. Microbiol., 13, 303 (1967)

20). T. Iguchi, T. Watanabe and I. Takeda: Agr. Biol. Chem., 31, 569, 574 (1967)

T. Iguchi, R. Kodaira and I.Takeda: Agr. Biol. Chem., 31, 885 (1967)

21). Kyowa Fermentation Industry Co., Ltd.: Fr. Pat., 1,473,629 (1967)

22). R.L. Raymond and J.B. Davis: Appl. Microbiol., 8, 329 (1960)

23). M.S. Taggart: U.S. Pat., 2,396,900 (1946)

24). A. Tanaka and S. Fukui: J. Ferment. Technol.,45, 611 (1967)

A. Tanaka, N. Ohishi and S. Fukui: J. Ferment. Technol., 45, 617 (1967)

25). K. Fujii, S. Shimizu and S. Fukui: J. Ferment. Technol., 44, 185 (1966)

S. Fukui, S. Shimizu and K. Fujii: J. Ferment. Technol., 45, 530 (1967)

26). A. Tanaka, T. Nagasaki, M. Inagawa and S. Fukui: J. Ferment. Technol., 46, 468 (1968)

A. Tanaka, T. Nagasaki and S. Fukui: J. Ferment. Technol., 46, 477 (1968)

27). A. Tanaka, H. Maki and S. Fukui: J. Ferment. Technol., 45, 1156, 1163 (1967)

28). A. Tanaka and S. Fukui: J. Ferment. Technol., (in press)

Synopsis

Part I. Production of vitamin B_6 by *Candida albicans* in hydrocarbon media

Vitamin B_6 production by hydrocarbon-utilizers was studied using a rotary shaker. Among the species including *Candida*, *Rhodotorula*, *Hansenula*, etc., *Candida albicans* exhibited the most excellent vitamin B_6-producing ability, i.e., 300 to 400 μg/l in a synthetic medium containing *n*-hexadecane as the sole carbon source. Addition of corn steep liquor and an appropriate non-ionic detergent such as Tween 85 or Span 60, enhanced the yeast growth and vitamin B_6 production. The cell yield as well as the vitamin formation was more increased as aeration or agitation was increased.

Production of vitamin B_6, especially pyridoxal phosphate, in a hydrocarbon medium was studied by cultivation of *Candida albicans* using a 10-l jar fermentor. When cultured in a 5 l medium consisting of *n*-hexadecane, salts, corn steep liquor, and Span 60, under vigorous aeration (10 l/min) and agitation (400 rpm), the yeast gave 3.96 g of dry cells per liter (after 4 days' culture), 604 μg/l of total vitamin B_6 (after 8 days) and 163 μg/l of pyridoxal phosphate (after 4 days).

The ratios of pyridoxal phosphate to total vitamin B_6 and to pyridoxal were on the 3rd. day of the cultivation 56 % and 85 %, respectively.

In the case of a glucose medium, the vitamin formed in the yeast cells was readily transferred into the supernatant even before reaching the maximal growth. In the hydrocarbon medium, in contrast, the relatively high content of vitamin B_6, especially of pyridoxal phosphate, in the cells was maintained after the maximal growth. In the cultured supernatant of the hydrocarbon fermentation, presence of pyridoxal phosphate was also ascertained by microbioassay and the enzymatic method.

A comparative study on the types of vitamin B_6 produced in the glucose medium and the hydrocarbon medium were performed using the bioautographic technique.

Part II. Production of cytochrome c by yeasts in hydrocarbon fermentation

It has been observed that oxidative degradation of hydrocarbons by microorganisms is carried out under more aerobic conditions than that of carbohydrates. Hence our attention was called to the question whether the yeasts grown

on hydrocarbons as a sole carbon source would produce a large amount of respiratory enzymes, especially cytochrome c. We also attempted to study on the relation among growth rate, cytochrome c content, and respiratory activity, and to use the cytochrome c content as an index of growth activity of yeast cells.

In Chapter 1, the effects of medium constituents, pH of culture medium, and aeration on cell growth were investigated using Candida albicans, which was screened as the typical hydrocarbon-utilizing yeast. Addition of corn steep liquor and a detergent, Tween 85, shortened the period of fermentation, and maintenance of pH at 5.2-5.4 increased the cell yield. The yield of dry cells was 5.25 g from 7.75 g of n-hexadecane when the yeast was cultured in the following medium; n-hexadecane 7.75 g, NH_4NO_3 5.0 g, KH_2PO_4 2.5 g, $MgSO_4.7H_2O$ 1.0 g, $FeCl_3.6H_2O$ 0.02 g, corn steep liquor 1.0 g, Tween 85 0.2 ml, and tap water 1000 ml. The effect of aeration or agitation was observed in the absence of detergent, but not in the presence of detergent, under the condition tested.

Chapter 2 deals with the study on the relations among the growth rate, cytochrome c content and respiratory activity of Candida albicans when the yeast was cultured on n-hexadecane under aeration.

Under the cultural condition employed, the maximal cytochrome c content was about 1.5 mg per g dry cells, which was analogous to that of glucose-grown cells. The growth rate was closely related with the cytochrome c content. The latter increased in parallel with the yeast growth, reached to the maximal value at the later part of the exponential phase, then falled off gradually. The correlationship between the cytochrome c content and the specific growth rate showed that the former can be used as an index of the growth activity of the yeast.

Oxygen-uptake of Candida albicans was inhibited by addition of sodium azide, and this suggested that the respiration of this yeast would proceed via an electron transport system including at least cytochrome a_1 and a_3. Also, we could detect cytochrome a, b, and c in reduced intact cells spectrophotometrically by the opal-glass method.

In Chapter 3, we determined the cytochrome c content of various yeasts grown on the "hydrocarbon mixture 2" (rich in n-undecane).

Part III. Production of carotenoids by Mycobacterium smegmatis in hydrocarbon media

During the course of the studies on the hydrocarbon utilization and vitamin B_{12} production by several kinds of bacteria the present author found out that some bacteria, such as Mycobacterium smegmatis, Nocardia lutea, and Nocardia corallina, grew well on n-alkanes and made up a clump of cells occluding hydrocarbon. In these cases, these bacteria produced orange to orange-red pigments easily extractable with n-hexane or n-hexane-acetone mixture, which were identified as mixtures of carotenoids.

Chapter 1 describes the studies on the effects of medium constituents, aeration, and addition of some organic compounds on the carotenoid production by Mycobacterium smegmatis. This bacterium could grow on n-alkanes of a medium chain-length (C_{11-19}), but not on those with shorter chains under the cultural condition used. Among pure n-alkanes, n-hexadecane was most suitable for the bacterial growth and carotenoid production, but "hydrocarbon mixture 2" (rich in n-undecane) was also suitable for carotenoid formation. Addition of surface detergent, Tween 80, and amino acids, such as glutamic acid, histidine, and serine stimulated the carotenoid production by Mycobacterium smegmatis, but some metal ions were inhibitory. Under the cultural conditions tested, the maximal carotenoid production was about 1 mg per liter of medium.

The isolation and characterization of carotenoids are mentioned in Chapter 2. These carotenoids produced by Mycobacterium smegmatis were classified into four categories; (1) intermediates in the carotenoid biosynthesis, such as phytofluene, ς-carotene, and neurosporene, (2) 4-keto-γ-carotene and its derivatives (containing eleven conjugated carbon-carbon double bonds and one conjugated carbonyl group), (3) derivatives of 3',4'-dehydro-4-keto-γ-carotene (containing twelve conjugated carbon-carbon double bonds and one conjugated carbonyl group), and (4) pigments having unknown chromophore. When carotenoids were extracted at the stationary phase of growth, the major part consisted of 4-keto-γ-carotene and its derivatives, possibly monohydroxy- and monomethoxy-4-keto-γ-carotene. Thus, we could isolate and characterize the precursors of 4-keto-γ-carotene, 4-keto-γ-carotene itself, and its derivatives, but could not detect γ-carotene or 4-hydroxy-γ-carotene, the presumable precursors of 4-keto-γ-carotene biosynthesis.

Electromicroscopical studies are carried out in Chapter 3, using hydrocarbon-grown and glucose-grown cells of Mycobacterium smegmatis. The carotenoid pigments of hydrocarbon-grown cells were more readily extracted by organic solvents compared to those of glucose-grown cells. This phenomenon

suggested that carotenoid pigment would have some role in the incorporation of hydrocarbon substrate into bacterial cells, and that the morphology, especially the construction of cell membrane, of hydrocarbon-grown cells would differ from that of glucose-grown cells. The participation of carotenoids in hydrocarbon uptake of bacterial cells could not be demonstrated, but some characteristics were shown about hydrocarbon-grown cells; that is, having round-shaped and somewhat obsecure cell membrane. Poly-β-hydroxybutylate-like substance was seen in the electromicrograph of hydrocarbon-grown cells as the low density area, but not in that of glucose-grown cells.

Part IV. Accumulation of porphyrin in culture filtrate of *Mycobacterium smegmatis* during hydrocarbon assimilation

During the course of the studies on the production of vitamin B_{12} and carotenoids by *Mycobacterium smegmatis*, we observed that this bacterium accumulated a relatively large amount of red pigment in the culture filtrate under the reduced aeration rather unfavourable to the cell growth and the carotenoid production in hydrocarbon fermentation.

The pigments extracted from the culture filtrate and from the bacterial cells were characterized as metal-free porphyrins

from
^their visible absorption spectra. Identification of methyl esters of these porphyrins was carried out by paper chromatography, and visible absorption-, infrared- and nuclear magnetic resonance spectroscopy. The red pigment extracted from the culture filtrate contained coproporphyrin III only, but the pigment extracted from the bacterial cells contained a small amount of unidentified porphyrin-like substance other than coproporphyrin III. The accumulation of porphyrin was observed under the reduced aeration (K_{La} = 5 hr^{-1}), but not under the larger aeration (K_{La} = above 28 hr^{-1}).

Part V. Substrate specificity of yeast in hydrocarbon fermentation

Although it is well known that yeast can assimilate n-alkanes, n-alkenes, and hydrocarbon mixtures, relative utilizability of n-alkanes has not been defined well. In the course of the studies on the utilization of hydrocarbons by several kinds of yeasts, we found out that Candida albicans, Candida intermedia, Candida tropicalis, and YH 101-C-1 (isolated from soil) could grow better on "hydrocarbon mixture 2" (rich in n-undecane) than on "hydrocarbon mixture 1" (rich in n-tridecane). Based on this fact, we investigated the substrate

specificity of Candida albicans using pure n-alkanes. Among the n-alkanes tested, n-decane, n-undecane, and n-dodecane were the best substrates, n-hexadecane was next to them, but n-tridecane, n-tetradecane, and n-pentadecane were inferior under the experimental conditions. n-Hexane and n-octane were not utilized by this yeast. In order to examine the relative utilizability of n-alkanes in the hydrocarbon medium containing a mixture of various hydrocarbons, we determined the residual substrates in the cultured broth using gas chromatographic technique.

The results obtained showed that the relative utilizability of n-alkanes in the hydrocarbon mixtures by Candida albicans was as follows; n-decane > n-undecane > n-dodecane > n-tridecane > n-tetradecane. A higher concentration of n-tridecane or n-tetradecane was rather inhibitory for the assimilation of n-decane or n-undecane. In the case of Candida tropicalis PK-233, similar results were obtained.

Part VI. Growth ability of yeasts and molds on hydrocarbons and their derivatives

Chapter 1 dealt with the growth ability of various yeasts on hydrocarbons, their mixtures, and their derivatives. The

yeasts which could not assimilate n-alkanes, could not also utilize their derivatives, such as primary alcohols or acids, as the sole source of carbon. The crude hydrocarbon mixtures, such as kerosene, light oil or wax, were not utilized by hydrocarbon-assimilating yeasts.

In Chapter 2, the capability of several kinds of molds to grow on hydrocarbons was investigated in three different cultural conditions: shaking culture, surface culture, and interface culture. Some strains of Aspergillus, Penicillium, Fusarium and Cladosporium could grow under these three conditions using "hydrocarbon mixture 1" (rich in n-tridecane) as sole carbon source. Among these conditions, surface culture was considered to be most suitable for the growth of molds. All these organisms could assimilate n-hexadecane and n-octadecane, but relative few could utilize light oil, kerosene, and/or paraffin (m.p. 42-44°C). Aspergillus flavus, Aspergillus niger, Penicillium notatum, and Cladosporium resinae, which could utilize n-alkanes of medium chain-length as carbon sources, could not assimilate n-alkanes of shorter chain-length (n-hexane and n-octane) and aromatic hydrocarbons so far tested.

Part I. Production of vitamin B_6 by *Candida albicans* in hydrocarbon media

1. Introduction

It would be important to produce some useful compounds from hydrocarbon substrates, and many investigations have been carried out on the production of cellular protein[1], amino acids[2,3,4], and nucleic acids[5], etc. Only a few studies, however, have been attempted to produce vitamins. These works dealt with vitamin B_2[6,7] and vitamin B_{12}[8], but concerning other important vitamins any papers have not been published.

In this part, we describe on the production of vitamin B_6 and its coenzyme forms from hydrocarbons, since it was known that some yeasts produced a relatively large amount of this vitamin, especially pyridoxal, in a carbohydrate medium[9].

Vitamin B_6, especially its coenzyme forms, i.e., pyridoxal phosphate and pyridoxamine phosphate, are the important vitamin participating in amino acid metabolism and amine oxidation in living bodies, and it will be useful to produce this vitamin from hydrocarbons by the fermentation technique.

From the study on the growth ability and vitamin B_6 productivity of various microorganisms using n-hexadecane as a sole carbon source, a yeast, Candida albicans was screened as a test organism, and the effects of medium constituents and cultural conditions were investigated about vitamin B_6 production.[10]

The types of the vitamin produced by this yeast were also examined using bioautographic technique[11], and the vitamin contents of bacteria and molds were investigated.[12,13]

2. Experimental procedure

2.1. Microorganism

The organism mainly used in this study was Candida albicans. Yeasts and molds were maintained on malt extract-agar slants, and bacteria were maintained on conventional natural nutrient-agar slants.

2.2. Cultivation method

For an inoculum, the yeast was cultured on malt extract-agar slant for 24 hrs at 30°C. A loopful of the cells was transferred into 100 ml of the medium containing 3.0 g of malt extract in a 500-ml shaking flask and cultured on a

Table 1. Composition of hydrocarbon medium used.

NH_4NO_3	5.0g
KH_2PO_4	2.5g
$MgSO_4.7H_2O$	1.0g
$FeCl_3.6H_2O$	0.02g
Corn steep liquor	1.0g
n-Hexadecane	10 ml
Tap water	1 ℓ
pH	5.2

Table 2. Composition of glucose medium used.

Glucose	16.5 g or 50.0 g
Peptone	5.0 g
Yeast extract	2.5 g
$(NH_4)_2SO_4$	6.0 g
KH_2PO_4	2.0 g
$MgSO_4.7H_2O$	0.5 g
$CaCl_2.2H_2O$	0.33 g
Distilled water	1 ℓ
pH	5.35

rotary shaker (220 rpm) at 30°C for 24 hrs. After centrifugation, cells were suspended into 25 ml of sterilized saline, and 1 ml of the cell suspension was added to 50 ml of the medium shown in Table 1 or 2, in a 500-ml shaking flask and cultured on a rotary shaker (220 rpm) at 30°C. Cultivation methods of bacteria and molds were shown in Part III and Part VI, respectively.

2.3. Extraction and bioassay of vitamin B_6

The cultured broth itself, or after being separated into

cells and supernatant by centrifugation, was hydrolized with 0.055 N H_2SO_4 for 1 hr at 120°C. By this procedure, bound type vitamers were converted to their free forms (pyridoxal, pyridoxine and pyridoxamine). The hydrolyzate was neutralized and diluted to appropriate volume after filtration. The amount of total vitamin B_6 was assayed by Saccharomyces carlsbergensis ATCC 4228 using pyridoxine hydrochloride as standard[14], and the content of pyridoxal was determined by Lactobacillus casei ATCC 7469[15].

2.4. Extraction of pyridoxal phosphate

After the centrifugation, yeast cells sedimented were treated with boiling water for 15 min and the mixture was centrifuged for 5 min at 3,000 rpm to obtain an extract. On the other hand, the cultured filtrate was neutralized and was treated at 100°C for 15 min. These extracts containing pyridoxal phosphate were assayed by enzymatic method as shown below.

2.5. Preparation of apotryptophanase

Escherichia coli Strain K was cultured with shaking (220 rpm) for 24 hrs at 30°C in a medium consisting of fish meat extract 10 g, peptone 10 g, NaCl 5 g and water 1,000 ml

(pH 7.0). The cells harvested were washed twice with chilled saline, sonicated for 10 min at 20 kc below 10°C, and then centrifuged for 10 min at 10,000 rpm under cooling. The supernatant thus obtained was acidified with diluted acetic acid to pH 4.7, allowed to stand at 0°C for 1 hr in the dark, and then centrifuged for 5 min at 8,000 rpm under cooling. The precipitate obtained was suspended in chilled water, dialyzed against cooled water overnight, then dissolved into 0.1 M potassium phosphate buffer, pH 7.5. The enzyme solution thus prepared was used for the assay of pyridoxal phosphate as apotryptophanase.

In some cases, the bacterial cells harvested were treated with acetone and stocked at 5°C. The acetone-dried cells were extracted overnight with cold water, and the crude extract was obtained after centrifugation[9]. This extract was also treated with diluted acetic acid as mentioned above.

2.6. Enzymatic assay of pyridoxal phosphate

Pyridoxal phosphate in the extracts obtained from the cultured broth was assayed by the enzymatic method recommended by Wada *et al.*[16], Sakamoto[17], and Snell *et al.*[18] The reaction mixture consisting of the original or diluted extract 1 ml, 0.02 M EDTA 0.5 ml and the enzyme solution 1.5 ml was

allowed to stand for 10 min at 37°C. The reaction was started by the addition of 0.01 M L-tryptophan solution 0.5 ml, which was preheated to 37°C, and was stopped with addition of 30 % trichloroacetic acid 0.5 ml after incubation at 37°C for 30 min. The reaction mixture was shaken vigorously with 5 ml of toluene for 5 min to extract indole formed. One mililiter of this toluene extract was pipetted out, allowed to stand for 20 min at room temperature after addition of 0.5 ml of 5.0 % p-dimethylaminobenzaldehyde in ethanol and 5 ml of 4 (v/v) % H_2SO_4 in ethanol, and then measured the optical density at 570 mμ. The calibration curve was prepared using authentic pyridoxal phosphate by the method mentioned above.

2.7. Paper chromatography, paper ionophoresis and bioautography

Paper chromatography was done by an ascending technique on Toyo Roshi No. 50 paper using Solvent A (tert-butanol-diethylamine-water-acetone 40:5:20:35) and water-saturated n-butanol as the developing solvents. Paper ionophoresis was carried out in 0.1 M acetate buffer, pH 5.2, at 500 V for 2 hrs. B_6-vitamers on the developed paper were detected by the bioautographic technique using Saccharomyces carlsbergensis ATCC 4228 and Lactobacillus casei ATCC 7469 as the test

organisms.

2.8. Purification of vitamin B_6 by phenol.

The hydrolyzate prepared as mentioned above was extracted with 85 % phenol. To this phenol layer, diethyl ether was added and the resulting water layer was used as a purified sample.

2.9. Separation of three types of vitamin B_6

The culture filtrate concentrated in vacuo was chromatographed on a paper using water-saturated n-butanol as a developer, and each B_6-vitamer was extracted with water from this chromatogram. Each extract was rechromatographed with Solvent A, and the zones showing activity to the test organism and a positive result on Gibbs test were extracted with water, and the extract was subjected to further purification by paper ionophoresis.

3. Results and discussion

3.1. Growth and vitamin B_6 productivity of yeast on hydrocarbon

The growth and the vitamin B_6 productivity were investi-

gated in a hydrocarbon medium using various kinds of yeast. The strains used were gifted from the Institute for Applied Microbiology, the University of Tokyo, the Institute for Fermentation, Osaka, and the Department of Fermentation Technology, Osaka University, or isolated from the natural sources. As shown in Table 3, although Candida albicans, Candida tropicalis, Candida lipolytica and YH 101-C-1 grew well on n-hexadecane, Candida albicans only produced a relatively large amount of vitamin B_6. Hence, we used this yeast in the following study.

3.2. Effects of medium constituents on growth and vitamin productivity of Candida albicans

3.2.1. Hydrocarbon

Under the cultural condition employed, n-hexadecane and n-octadecane were good carbon sources, but kerosene and light oil were hardly utilized (Table 4).

3.2.2. Nitrogen source

Nitrogen source is an important factor for the growth of microorganisms because of its utilizability and the variation of pH attributed to the residual anion. Therefore, various nitrogen compounds were added to the medium shown in Table 1 with exception of NH_4NO_3, at the concentration

Table 3. Growth and vitamin B_6 production by several yeast in a hydrocarbon medium (Shaking culture).

Yeast		Cell yield (g dry wt./l)	Total vit. B_6 produced (μg/l)	(μg/g dry cell)
Candida albicans	IFO 0583	2.78	368	132
Candida lipolytica	IFO 0717	1.46	120	82
Candida rugosa	IFO 0591	0.74	37	50
Candida tropicalis	IFO 0589	2.63	51	19
Candida utilis	IFO 0619	0.23	34	148
YH 101 C1 (isolated from soil)		2.83	48	17
Rhodotorula rubra	IFO 0382	0.17	27	159
Hansenula anomala	IFO 0127	0.29	34	117

Cultivation was carried out on a rotary shaker (220 rpm) for 7 days at 30°C.

Table 4. Growth of C. albicans on various hydrocarbons.

Hydrocarbon	Amts. added (g or ml/l)	Cell yield (g dry wt./l)
n-Hexadecane	10 ml	3.14
Cetyl alcohol	10 g	2.06
n-Octadecane	10 "	2.30
Stearyl alcohol	10 "	0.64
Stearic acid	10 "	0.60
Paraffin (mp. 42-44°C)	10 "	0.52
Wax	10 "	0.42
Kerosene	10 ml	0.18
Light oil	10 "	0.24

Cultural conditions were the same as those in Table 3.

of 0.2 and 0.5 %, respectively. As shown in Table 5, the yeast grew well at the higher concentration of nitrogen source except casamino acid. Most suitable nitrogen compound was $(NH_4)H_2PO_4$ for the growth rate and cell yield of this yeast (Fig. 1), but for vitamin production, especially pyridoxal formation, NH_4NO_3 was most excellent (Table 5). With the addition of casamino acid or urea, the yeast showed a two-step growth (Fig. 1). This phenomenon would be explained by considering that these compounds were consumed as a carbon source at the initial stage of the fermentation and afterward the carbon of hydrocarbon substrate was utilized.

3.2.3. Natural nutrient

Although Candida albicans did not seem to require any special organic compound for its growth, we expected a effect of some natural nutrient on the vitamin production in the hydrocarbon medium without any organic nitrogen or natural nutrients. The yeast growth was stimulated by the addition of corn steep liquor or yeast extract as seen in Fig. 2, while the vitamin production was increased by the addition of corn steep liquor or casamino acid (Table 6). From these results, we used corn steep liquor as the additive to the hydrocarbon medium.

3.2.4. Detergent

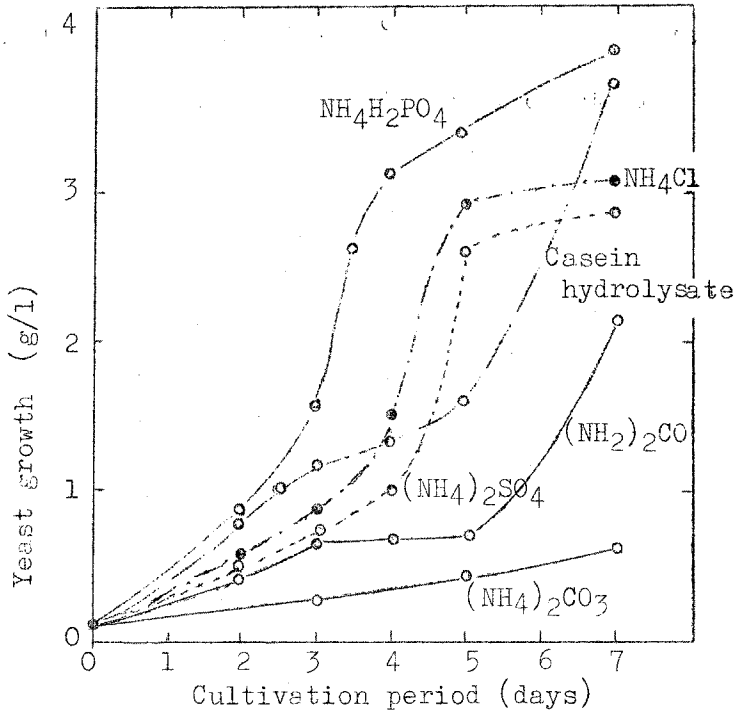


Fig.1. Effect of nitrogen sources on the growth of C.albicans. Cultivation was carried out at 30°C on a rotary shaker (220 rpm) The conc. of casamino acid was 0.2%(w/v)and the conc. of other nitrogen compounds were 0.5%.

Table 5. Effect of nitrogen sources on the yeast growth and vitamin B_6 production in a hydrocarbon medium.

Nitrogen source	Amts.added (w/v %)	Cell yield (g dry wt/l)	Total vit. B_6 produced (μg/l)	PAL produced (μg/l)
NH_4NO_3	0.2	0.92	-	-
	0.5	3.19	478	173
$(NH_4)H_2PO_4$	0.2	2.03	-	-
	0.5	3.93	267	72
$(NH_4)_2SO_4$	0.2	0.47	-	-
	0.5	2.85	201	88
$(NH_4)_2CO_3$	0.2	0.46	-	-
	0.5	0.62	27	12
NH_4Cl	0.2	0.54	-	-
	0.5	3.06	247	89
$(NH_2)_2CO$	0.2	1.23	-	-
	0.5	2.16	111	32
Casamino acid	0.2	3.73	177	88
	0.5	2.03	-	-

Cultivation was carried out on a rotary shaker (220 rpm) for 7 days at 30°C with 500 ml flask containing 50 ml medium.

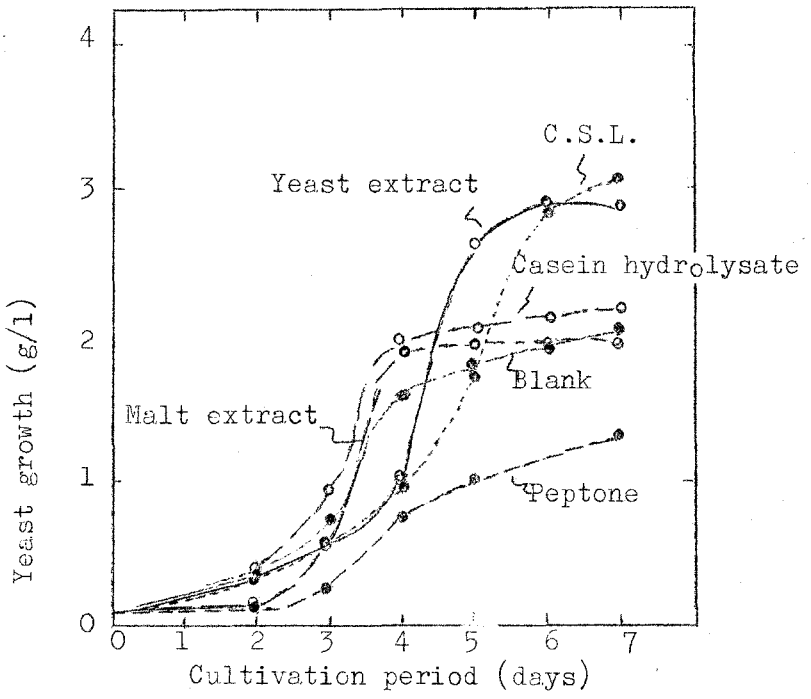


Fig.2. Effect of natural nutrients on the growth of C.albicans. Cultivation was carried out at 30°C on a rotary shaker (220 rpm).

Table 6. Effect of natural nutrients on the yeast growth and vitamin B_6 production.

Natural nutrients	Amts.added (mg/l)	Cell yield (g dry wt./l)	Total vit. B_6 produced(μg/l)	PAL produced (μg/l)
No addition	-	2.00	352	121
Corn steep liquor	100	1.93	-	-
	1000	3.09	464	150
Yeast extract	100	1.93	-	-
	1000	2.87	293	107
Malt extract	100	1.97	170	99
	1000	1.90	-	-
Fish extract	100	1.49	276	87
	1000	1.40	-	-
Peptone	100	1.33	54	40
	1000	0.65	-	-
Casamino acid	100	2.20	408	151
	1000	1.98	-	-

Cultural conditions were the same as those in Table 5.

It was expected that a surface detergent would stimulate the emulsification of a hydrocarbon and would increase the growth rate of the yeast as the result. Hence, we investigated the effect of non-ionic detergent and found out that most of them were effective to the growth and vitamin B_6 productivity of Candida albicans. Especially, Tween 85 and Span 60 were superior to both purposes, as seen in Table 7.

3.3. Effect of aeration or agitation

Utilization of hydrocarbons by microorganisms was usually performed under aerobic conditions, and so the effect of aeration or agitation on the vitamin production by this yeast was investigated by varying the volume of medium in 500-ml shaking flasks. As the volume of medium in the flask was small, that is the aeration was large, the cell yield as well as the vitamin production was enhanced. No growth was seen in a flask containing more than 100 ml of medium. But the vitamin productivity of unit cell mass was nearly the same below 75 ml of medium, as shown in Table 8. Thus, it appears necessary to increase the cell yield in order to produce a larger amount of vitamin B_6 in hydrocarbon fermentation. From the comparative study on the media with or without a

Table 7. Effect of detergents on vitamin B_6 production

Detergent	Amts. added (mg or μl/l)	Cell yield (g/l)	Total vit. B_6 produced(μg/l)	PAL produced (μg/l)
Tween 20	4 μl	2.42	552	308
Tween 40	4 μl	2.45	650	271
Tween 60	4 μl	2.42	658	269
Tween 80	4 μl	2.34	322	208
Tween 85	40 μl	3.24	972	184
Span 20	4 μl	2.78	504	320
Span 40	4 mg	2.82	608	267
Span 60	4 mg	2.85	916	397
Span 80	4 μl	2.67	530	289
None	0	2.72	386	230

Cultural conditions were the same as those in Table 5.

detergent, it was shown that the emulsification of hydrocarbons, rather than the supply of oxygen, was more important factor for the growth of this yeast under the cultural condition used. That is, the agitation was the limiting factor for the growth.

3.4. Time course of vitamin B_6 production by Candida albicans

This section deals with a comparative study on time course changes in vitamin B_6 production, distribution of the vitamin between the cells and the culture filtrate, and the ratio of pyridoxal to total forms of vitamin B_6 when Candida albicans was cultured on hydrocarbon media and glucose media. The results obtained were summarized in Table 9.

3.4.1. Variation of amount of total vitamin B_6

In the glucose media, the amount of vitamin B_6 produced increased along with the yeast growth till the cell yield reached its maximum, then decreased rapidly during the stationary growth phase. In this case, intracellular vitamin reduced suddenly at the stationary phase, but extracellular one did not decrease even at this phase of cultivation. The results suggested that the biosynthesis and the excretion would occur simultaneously before the stationary phase and that at the stationary phase, the intracellular vitamin would be excreted from the cells into the culture filtrate and be degraded at this place.

On the other hand, the total amount of vitamin B_6 produced in hydrocarbon media also increased along with the yeast growth but did not decrease during the stationary phase. Extracellular vitamin rather increased at this phase, and this result suggested that vitamin synthesis and excretion would

Table 8. Relationship between oxygen supply and vitamin B_6 production by C. albicans.

Amt. of medium in 500 ml flask (ml)	Kd ($\times 10^{-6}$)	Cell yield (g dry wt./l)	Vit. B_6 produced	
			in total broth ($\mu g/l$)	per unit cell ($\mu g/g$ dry cell)
25	7.4	3.13	500	160
50	5.2	2.78	368	132
75	4.0	2.33	339	146
100	3.8	0.25	0	0

Table 9. Time course of vitamin B_6 production by C. albicans in a hydrocarbon medium or in glucose media.

Medium	Hydrocarbon medium						Glucose medium (5% glucose)				Glucose medium (1.65% glucose)			
Cultivation time (days)	2	3	5	6	7	8	1	2	3	5	10 (hrs)	1	2	3
Cell yield (g dry wt./l)	0.37	0.38	0.39	2.55	2.78	2.77	8.30	10.9	11.3	10.7	3.66	5.65	6.10	6.00
Vitamin B_6 produced														
in total cells (µg/l)														
total B_6	21	23	32	60	70	78	100	428	228	57	189	292	245	220
PAL	17	18	20	32	48	44	81	396	167	19	...	...	...	...
in supernatant (µg/l)														
total B_6	trace	trace	trace	111	147	256	49	492	1000	1000	101	281	481	577
PAL	"	"	"	78	105	120	40	272	458	393	...	...	...	...
Sum (µg/l)														
total B_6	21	23	32	172	217	334	149	920	1228	1057	290	573	726	799
PAL	17	18	20	110	153	164	121	668	625	412	...	...	...	...
Amts. produced per unit wt. cell (µg/g)														
total B_6	56.7	60.5	82.1	23.5	25.2	28.2	12.0	39.2	20.1	5.3	51.6	51.7	40.1	36.7
PAL	45.9	47.4	51.3	12.5	17.3	15.9	9.8	36.3	14.8	1.8	...	...	...	...
Distribution of														
in supernatant/in cell														
total B_6	-	-	-	1.85	2.1	3.3	0.49	1.2	4.4	17.6	0.54	0.96	1.96	2.62
PAL	-	-	-	2.4	2.2	2.7	0.49	0.7	2.7	20.7	...	...	...	...
Ratio of PAL/total B_6	80	78	63	64	67	49	79	73	51	39	-	-	-	-

... not assayed - not calculated

Table 10. Time course of vitamin B_6 production by C. albicans in a hexadecane-medium.

Cultivation time (days)		2	3	4	5	6	7	8
Cell yield (g dry cell/l)		0.41	3.00	3.96	3.86	3.39	3.34	3.54
pH of broth		5.2	2.6	2.1	2.3	2.4	2.1	2.1
Vitamin B_6 produced in cells (μg/l)	Total B_6	51	157	151	151	55	20	51
	PAL	40	98	102	92	28	16	16
	PAL-P	-	86	82	74	26	13	11
In supernatant (μg/l)	Total B_6	53	133	241	359	388	457	553
	PAL	16	92	118	93	94	86	77
	PAL-P	-	76	81	64	79	55	62
Total (μg/l)	Total B_6	104	290	392	510	443	479	604
	PAL	56	190	220	185	122	102	93
	PAL-P	-	162	163	138	105	68	73
Vit. B_6 per cells (μg/g dry cell)	Total B_6	254	97	99	132	131	143	171
	PAL	137	63	56	48	36	31	26
	PAL-P	-	54	41	36	31	20	20
Distribution of vit. B_6 in supernatant and in cells	Total B_6	1.04	0.85	1.60	3.38	8.05	23.95	10.81
	PAL	0.40	0.94	1.16	1.01	3.36	5.38	4.81
	PAL-P	-	0.88	0.99	0.91	3.04	4.23	5.64
Percentage of PAL to total B_6%		54	66	56	36	28	21	15
Percentage of PAL-P to PAL%		-	85	74	74	86	67	78

Cultivation was carried out at 30°C using a 10 l-jar fermentor containing 5 l of medium and an aeration rate was 10 l/min. The PAL-P contents in the samples were assayed enzymatically using apotryptophanase after extraction with hot water.

continue even at the stationary phase and that the apparent amount of the intracellular vitamin would be retained at a definite level for relatively long cultivation period.

This difference between the glucose and the hydrocarbon media may be attribute to the difference of the assimilation rates of the carbon source, especially the degradation rates of the metabolic intermediates of the carbon substrates; that is, higher alcohol or higher fatty acid produced as metabolic intermediate of hydrocarbon may serve as a carbon source of the vitamin synthesis.

3.4.2. Variation of amount of pyridoxal produced

In the glucose medium, the amount of total pyridoxal formed falled off slightly at the stationary phase. This was arised from the decrease of intracellular pyridoxal, since extracellular one showed only a little reduction. The variation of pyridoxal was relatively in concert with that of total vitamin B_6, excepted that the amount of pyridoxal reached to the maximum in two days' cultivation.

On the other hand, the amount of total pyridoxal produced in the hydrocarbon medium was slightly increasing even on the eighth day of cultivation, but intracellular one began to fall off at this time. Similar to the case of total vitamin B_6, pyridoxal formed in hydrocarbon fermentation retained in the

yeast cells for longer period than in glucose fermentation.

3.4.3. Variation of ratio of pyridoxal to total vitamin B_6

In glucose medium, the ratio of pyridoxal to total vitamin was 73 % on the second day, but decreased to 51 % on the third day when the cell yield reached to the maximum, and then falled off rapidly. On the other hand, this ratio was 67 % on the seventh day when the yeast showed the maximal yield, followed by the decrease, in the hydrocarbon medium.

The decrease of the ratio of pyridoxal to total vitamin was resulted from the change of pyridoxal phosphate, which participated as the coenzyme of various amino acid metabolism in the organism, into pyridoxamine or pyridoxine.

3.4.4. Relation between cell growth and vitamin B_6 production

As mentioned above, the growth rate and the cell yield were rather smaller in the hydrocarbon medium, but the amount of vitamin produced per unit cells was almost constantly 110-120 $\mu g/g$ dry cells at the maximal growth in either medium used. From this fact, we can conclude that it will be most important factor for the vitamin B_6 production to stimulate the yeast growth.

3.5. Vitamin B_6 production in aerated culture

The cultivation of Candida albicans was carried out in

a 10-l jar fermentor containing 5 l of the hydrocarbon medium supplemented with 20 mg of Span 60. As shown in Table 10 and Fig. 3, the contents of total vitamin B_6, pyridoxal and pyridoxal phosphate in the yeast cells were sustained at their maximal levels from third to fifth day of cultivation, although the cell yield reached to the maximum on the fourth day. This phonomenon was one of the characteristics of the hydrocarbon-grown cells and would be attributed to the assimilation rate of the carbon substrate or its permeability of cell membrane. Total vitamin in the culture filtrate increased till eighth day when the yeast showed no more growth. The amounts of pyridoxal and its phosphate ester in the supernatant were nearly constant during the stationary phase of growth, and this fact suggested that pyridoxal phosphate formed in the yeast cells would be excreted into the culture filtrate followed by the degradation into its free form and then to other forms.

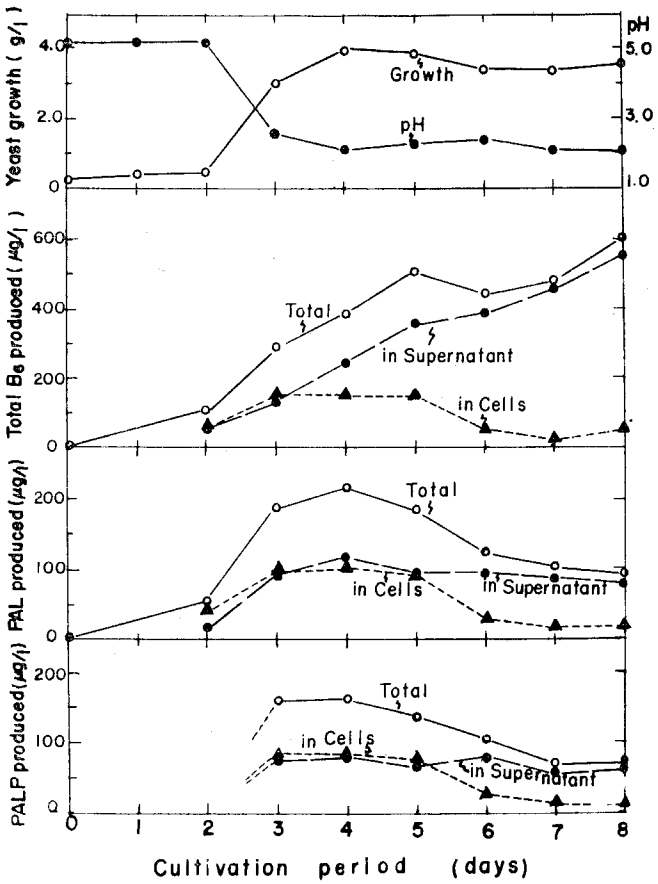


Fig. 3. Time course on the growth of C. albicans and vitamin B_6 production in a hydrocarbon medium.

3.6. Types of vitamin B_6 produced in shaking culture

3.6.1. Cultivation on glucose

The types of vitamin B_6 produced in the glucose medium were investigated using one day's culture when the vitamin was being accumulating and the three days' culture when the vitamin was being excreting. In the cells of one day's culture pyridoxal and pyridoxamine were detected as the free forms, but pyridoxine was found in neither free form nor its phosphate ester form as shown in Fig. 4. In three days' culture, pyridoxamine only was detected as free form, while pyridoxal and pyridoxine existed as their coenzyme forms in the cells. On the contrary, the three types were all detected as free forms in the supernatant and the activity of pyridoxine was largest. Although the activity of pyridoxal increased after hydrolysis, the excretion of pyridoxal phosphate was not yet confirmed about the glucose-grown Candida cells.

3.6.2. Cultivation on hydrocarbon

The types of vitamin B_6 produced in the hydrocarbon medium were investigated using five days' culture which was growing exponentially and seven days' one which was in the stationary phase of growth.

In the cells of either culture phase, pyridoxal and pyridoxamine were detected as both free formes and ester

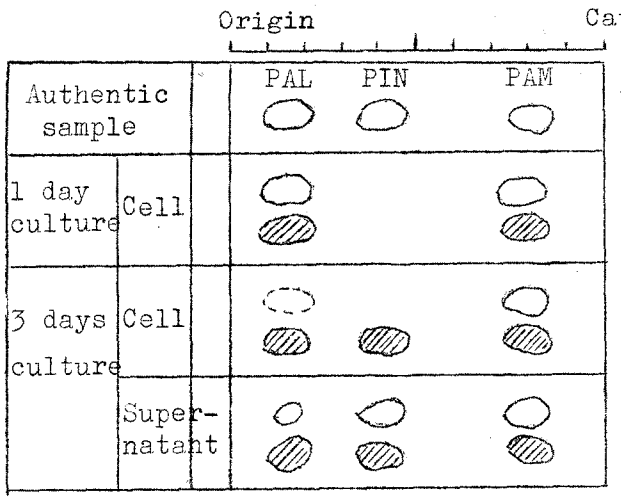


Fig. 4. Bioautogram of vitamin B_6 produced by C. albicans in glucose medium.

Paper electrophoresis was carried out in acetate buffer (pH5.2)

Test organism: Sacch. carlsbergensis 4228

Before hydrolysis

After hydrolysis

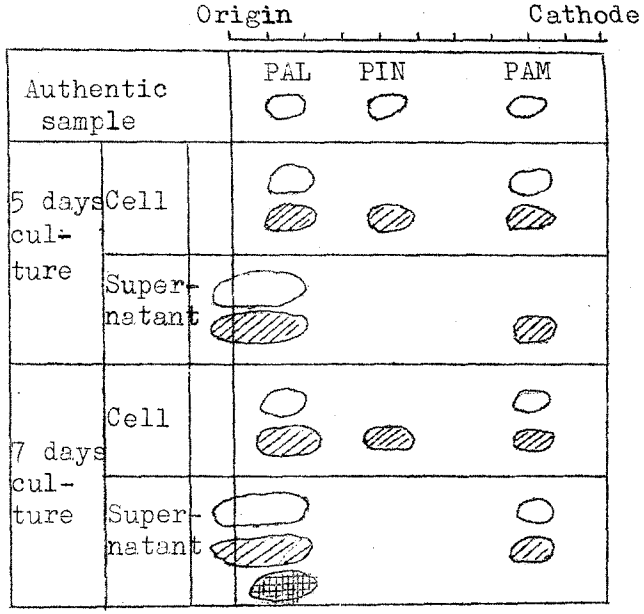


Fig. 5. Bioautogram of vitamin B_6 produced by C. albicans in hexadecane medium.

Paper electrophoresis was carried out in acetate buffer (pH5.2).

Test organism: Sacch. carlsbergensis 4228 (&) and Lactobac. casei ( )

Before hydrolysis.

After hydrolysis.

Before and after hydrolysis, respectively.

forms, but pyridoxine existed only as phosphate ester (Fig. 5). In the supernatants, although pyridoxamine was detected as both forms, pyridoxine was not detected. The active zone corresponding to pyridoxal on the paper ionophoresis was rather larger when assayed by Saccharomyces carlsbergensis (for all free forms) than when bioautographed using Lactobacillus casei (for pyridoxal only). This fact suggested that the zone corresponding to pyridoxal would be including other B_6-vitamer, pyridoxine, and that this phenomenon would be attributed to the contamination of a certain impurity occured in the supernatant. The existence of the impurity was confirmed when the various samples from the supernatant were chromatographed on a paper, as seen in Fig. 6. This impurity interacted with pyridoxal and pyridoxine, and their R_f values were lowered as the result. The active zones containing both vitamin were purified into individual components by means of paper chromatography and paper ionophoresis, and the identification of each vitamin was summarized in Table 11. From this result, it was confirmed that pyridoxine and pyridoxal, as well as pyridoxamine, were included in the culture filtrate of the hydrocarbon media as the free forms.

In Fig. 7, the absorption spectra of purified pyridoxine were shown under the acidic, neutral and basic conditions.

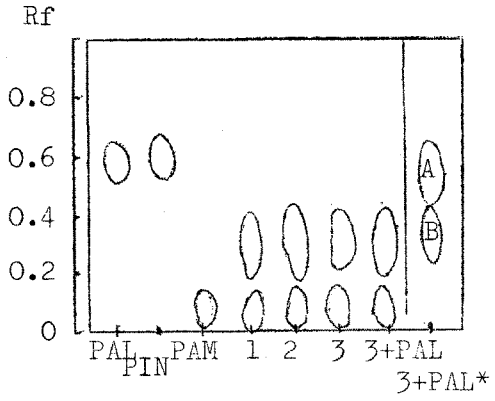


Fig.6. Bioautogram of vitamin B_6 produced by C.albicans in hexadecane medium.

Solvent: water saturated n-butanol
Test organism: Sacch. carlsbergensis 4228
Sample 1: Concentrated cultured supernatant
2: Hydrolysate of 1
3: Phenol-purificate of 1
3+PAL* 200 fold PAL was added to 3, and detected by fluorescence.
A: fluorescent substance(s) in the broth.
B: PAL

Table 11. R_f and R_{PAM} values of vitamin B_6 produced by C. albicans in a hexadecane medium.

	R_f value on paper chromatogram		R_{PAM} value on paper electrophoresis*
	Water satd. butanol	Solvent A**	
Authentic PAL	0.59	0.78	0.20
PAM	0.09	0.69	1.00
PIN	0.62	0.50	0.52
1	0.07,0.17~0.46	0.45~0.69	-0.10~0.25,1.00
4	0.09	0.71	1.00
5	0.60	0.50,0.81,0.95	0.20,0.52
5+PAL	0.60	0.50,0.81,0.95	0.20,0.52

R_f and R_{PAM} values were the results of bioautograms using Sacch. carlsbergensis.
* Paper electrophoresis was carried out in 0.1M acetate buffer, pH5.2
**Solvent A: t-butanol·diethylamine·water·acetone (40:5:20:35).
Sample 1: Concentrated cultured supernatant (7 day's).
Sample 4: Lower vitamin B_6 substance of sample 1 developed with water satd. n-butanol.
Sample 5: Upper vitamin B_6 substance of sample 1 developed with water satd. n-butanol.

These were good agreed with those of the authentic one. Other vitamers could not be completely separated from a contaminant.

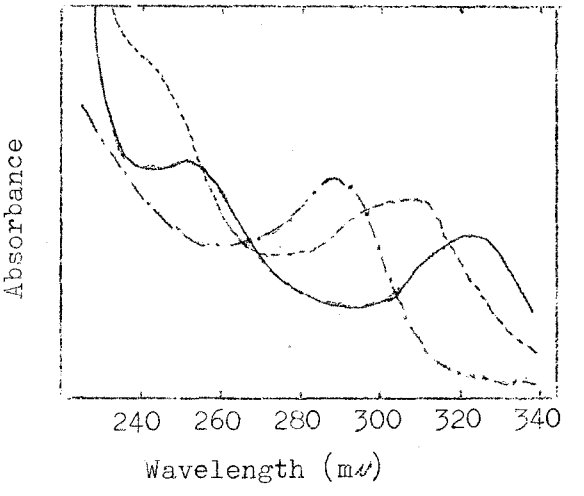


Fig. 7. Ultra-violet absorption spectra of pyridoxine obtained from cultured broth of C. albicans.

The spectra were measured in neutral (——), acidic (— ——), and basic (...) conditions.

3.7. Production of vitamin B_6 by other organisms

Vitamin B_6 productivity of other microorganisms were investigated using hydrocarbon media. Some molds including Aspergillus niger, Cladosporium resinae, Penicillium chrysogenum and Penicillium notatum, and an unidentified bacterium, No. 4313, produced relatively large amount of vitamin B_6 comparable with that of Candida albicans, as shown in Table 12 and 13. The vitamin contents of other organisms tested were rather smaller, although they showed fairly good growth on n-hexadecane.

Table 12. Vitamin B_6 production by bacteria

Bacterium	Vit. B_6 produced (μg/l)
Corynebacterium simplex	96
Mycobacterium smegmatis	216
Nocardia corallina	31
Nocardia gardoneri	117
Nocardia lutea	41
No. 4313	429

Bacteria were cultured on n-hexadecane with shaking for 7 to 9 days.

Table 13. Vitamin B_6 production by molds

Mold	Vit. B_6 produced (μg/l)
Aspergillus flavus	7
Aspergillus fumigatus	42
Aspergillus niger	383
Aspergillus oryzae	10
Aspergillus terreus	52
Cladosporium resinae	367
Fusarium moniliforme	55
Fusarium oxysporum	41
Fusarium solani	44
Penicillium chrysogenum	365
Penicillium notatum	378
MH 201	5

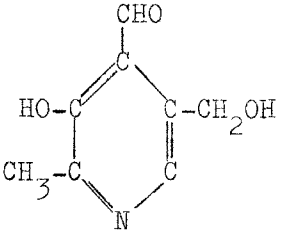
Molds were cultured statically on n-hexadecane for 14 days.

4. Summary

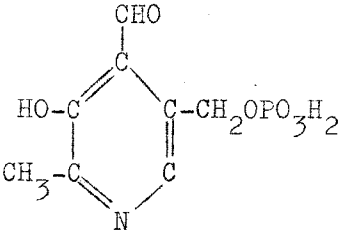
The growth ability and the vitamin B_6 productivity of various microorganisms were investigated using n-hexadecane as a sole carbon source. From the results obtained, a yeast, Candida albicans was chosen as the test organism, and was studied on the vitamin B_6 productivity under the various cultural conditions. This yeast could grow well on n-hexadecane and n-octadecane, but could not on kerosene or light oil. The vitamin production was stimulated by the addition of corn steep liquor and a certain detergent upto 1 mg per liter. The growth rate, cell yield and vitamin production in the hydrocarbon medium were rather smaller than those in the glucose medium, but the specific content of the vitamin in the cells was about 100-120 $\mu g/g$ dry cells in either medium. The yeast grown in the hydrocarbon medium retained the vitamin within their cells for a longer period than that grown in the glucose medium. This is the one of the characteristics of hydrocarbon fermentation. Another characteristics of hydrocarbon-grown cells was the excretion of pyridoxal phosphate into the culture filtrate. The formation of vitamin B_6, especially of pyridoxal phosphate, continued after the maximal cell growth followed by the excretion of this vitamin in the supernatant even at the rather late culture phase.

The types of vitamin B_6 produced in the both media were nearly same when being detected by paper chromatography and paper ionophoresis followed by bioautography.

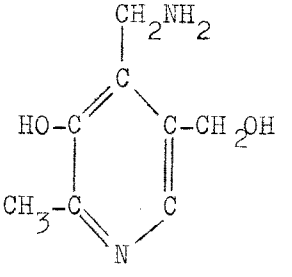
5. Chemical structures and abbreviations of B_6-vitamers



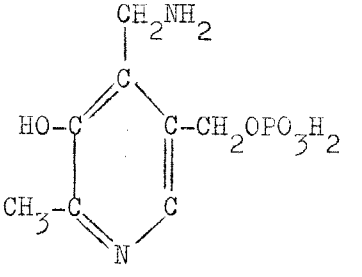
Pyridoxal
(PAL)



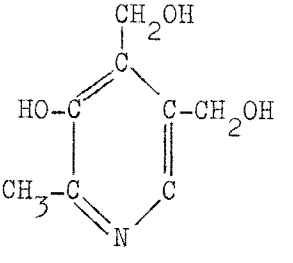
Pyridoxal phosphate
(PAL-P)



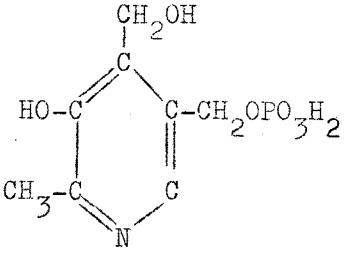
Pyridoxamine
(PAM)



Pyridoxamine phosphate
(PAM-P)



Pyridoxine
(PIN)



Pyridoxine phosphate
(PIN-P)

6. References

1). A. Champagnat, C. Vernet, B. Laine and J. Filosa: Nature, 197, 13 (1963)

2). Y. Imada, E.C. Pantskhava and K. Yamada: Amino acid. Nucleic acid, 16, 56 (1967)

3). T. Iguchi, I. Takeda and S. Senoh: Agr. Biol. Chem., 29, 589 (1965)

4). R. Uchino, S. Otsuka and I. Shiio: J. Gen. Appl. Microbiol., 13, 303 (1967)

5). T. Iguchi, T. Watanabe and I. Takeda: Agr. Biol. Chem., 31, 569, 574 (1967)

6). Y. Suzuki, T. Matsubayashi and T. Mochizuki: Proc. of the 37th Annual Meeting of Agr. Chem. Soc., Japan (Kyoto) (1966)

7). T. Sato, A. Maekawa, T. Suzuki and K. Sahashi: Vitamins (Kyoto), 34, 542 (1966)

8). K. Fujii, S. Shimizu and S. Fukui: J. Ferment. Technol., 44, 185 (1966)

S. Fukui, S. Shimizu and K. Fujii: J. Ferment. Technol., 45, 530 (1967)

9). T. Sato: Vitamins (Kyoto), 29, 516, 519 (1964)

10). A. Tanaka and S. Fukui: J. Ferment. Technol., 45, 611(1967)

11). A. Tanaka, N. Ohishi and S. Fukui: J. Ferment. Technol., 45, 617 (1967)

12). A. Tanaka, S. Shimizu and S. Fukui: J. Ferment. Technol., 46, 461 (1968)

13). S. Fukui and A. Tanaka: Hakko Kyokai-Shi, 26, 19 (1968)

14). S. Fukui, T. Kishibe and Y. Tani: Vitamins (Kyoto), 6, 436 (1953)

Y. Tani: Vitamins (Kyoto), 12, 546 (1957)

15). J.C. Rabinowitz, N.I. Mondy and E.E. Snell: J. Biol. Chem., 175, 147 (1948)

16). H. Wada, T. Morisue, Y. Sakamoto and K. Ichihara: J. Vitaminol., 3, 183 (1957)

17). Y. Sakamoto: Vitamins (Kyoto), 24, 232 (1961)

18). D. McCormick, M. Gregory and E.E. Snell: J. Biol. Chem., 236, 2076 (1961)

Part II. Production of cytochrome c by yeasts in hydrocarbon fermentation

Chapter 1. Production of yeast cells from hydrocarbon

1. Introduction

The production of microbial cells, useful cell materials or metabolites from hydrocarbons is one of the most up-to-date problem in the field of fermentation technology. The production of cytochrome c, which is an important respiratory enzyme and can be used for medicine, is an interesting object since the assimilation of hydrocarbons by microorganisms is carried out under aerobic condition. The productivity of cytochrome c by yeast was supposed to be concerted with the cell growth, and therefore, we investigated the yeast growth on n-hexadecane at first.

In this chapter, the effect of medium constituent, pH of culture medium, and aeration degree on the growth of Candida albicans were dealt.

2. Experimental procedure

2.1. Microorganism

The organism used in this study was Candida albicans Berkhout IFO 0583. This yeast was maintained on malt extract-agar slant.

2.2. Cultivation method

Each 500-ml shaking flask contained 100 ml of medium as shown in Table 1. Other cultural conditions were same as those in Part I.

2.3. Determination of cell yield

Determination of cell yield was carried out as follows; 1 ml of cultured broth was centrifuged and then washed with n-hexane and water, successively, and suspended into 25 ml of water then measured by optical density at 570 mμ, and dry cell yield was determined from the calibration curve shown in Fig. 1.

Other experimental conditions were shown in the results, respectively.

3. Results

3.1. Effect of various carbon sources on cell yield

As mentioned in Part I, Candida albicans could assimi-

Table 1. Composition of hydrocarbon medium used.

NH_4NO_3		5.0 g
KH_2PO_4		2.5 g
$MgSO_4 \cdot 7H_2O$		1.0 g
$FeCl_3 \cdot 6H_2O$		0.02 g
Corn steep liquor		1.0 g
Tween 85		0.2 ml
n-Hexadecane		10.0 ml
Tap water		1000 ml
pH	5.2-5.4	

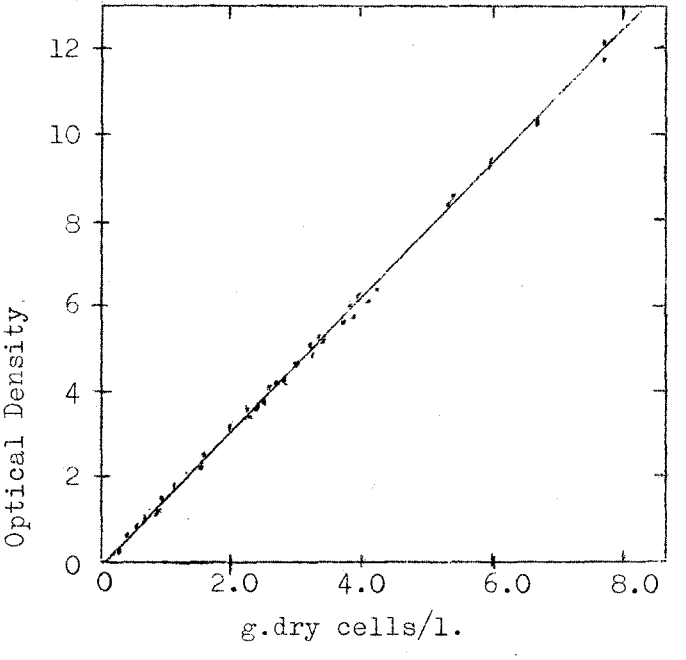


Fig. 1. Calibration curve of dry cell yield.
Optical density was measured at 570 mμ.

late n-hexadecane and n-octadecane, as a sole carbon source, but hardly hydrocarbon mixtures, such as kerosene, light oil, paraffin and wax. The ability of this organism to assimilate n-alkanes of C_{10-20} and their mixtures was described in Part V.

3.2. Effect of detergent.

By addition of detergent into medium, emulsification of hydrocarbon will occur and be stabilized, and subsequently, the contact surface of hydrocarbon with cells will increase and the growth rate of yeast will be stimulated. In Part I, we described that some non-ionic detergents, such as Tween 85 and Span 60, stimulated the rate of growth but did not affect the final cell yield. In this experiment, we used Tween 85 and investigated its effect on the fermentation period of Candida albicans. As shown in Fig. 2A, as the concentration of detergent increased, the period of cultivation was shortened. Cell yield was not affected, but when high concentration of tween 85 was added, yeast cells autolyzed vigorously after the maximum growth. To obtain the theoretical minimal fermentation period, the amount of Tween 85 added vs. the fermentation period were plotted reciprocally. As shown in Fig. 2B, the relation between these two reciprocals was linear, and expressed by the following equation;

$$T = 140 - \frac{100\ X}{25 + 0.95\ X}$$

Where,

T = the fermentation period (hr)

X = amount of Tween 85 added (μ l/l of medium)

The theoretical minimal fermentation period was calculated as 35 hrs under the experimental condition used when Tween 85 would be added by infinite amount. Herefrom, we used 200 μl/l of Tween 85 in the subsequent experiments, since the addition of this amount of detergent reduced the fermentation period to about 40 hrs, greatly shorter than in the case of no addition (140 hrs). Further addition of detergent would stimulate autolysis of yeast cells.

3.3. Effect of aeration

Fermentation of hydrocarbons which requires vigorous aeration will be affected by the amount of oxygen dissolved or supplied into culture broth. However, from the view of biochemical engineering, one hopes that the rate of aeration and agitation are as little as possible.

We examined the effects of aeration and agitation by varying the amounts of medium in 500-ml shaking flasks from 25 to 150 ml, with and without Tween 85. Without Tween 85,

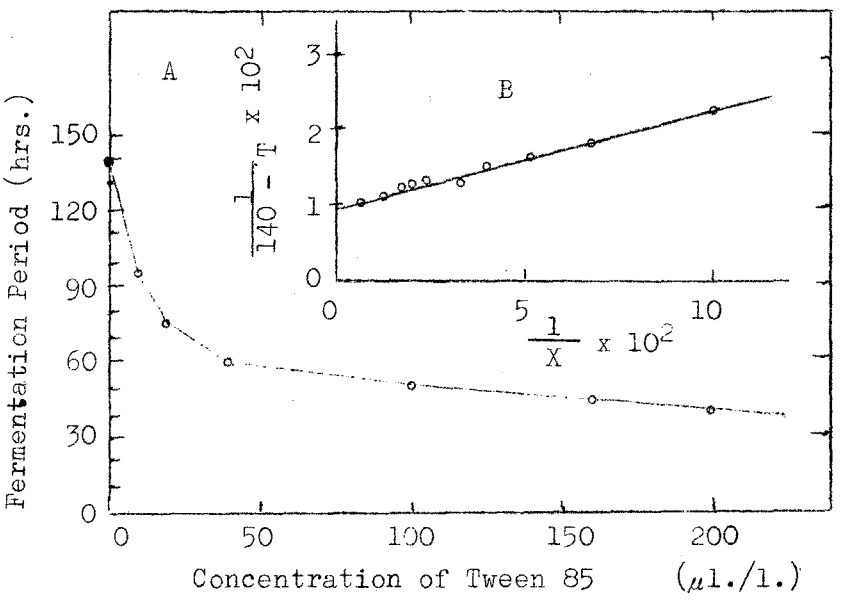


Fig. 2. Effect of concentration of Tween 85 on fermentation period.

Curve A: relation between concentration of Tween 85 and fermentation period.

Curve B: reciprocal relation between concentration of Tween 85 and fermentation period, where, X=concentration of Tween 85 (μl/l) and T=fermentation period (hr).

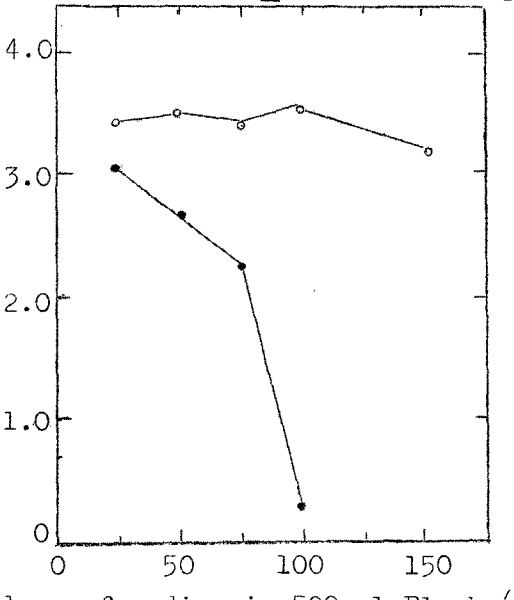


Fig.3. Effect of oxygen-supply on cell yield.

Cultivation was carried out on a rotary shaker (220 rpm) at 30°C
○ - ○; with Tween 85(200 μl/l
● - ●; without Tween 85.

effect of aeration and agitation were remarkable, and no growth was observed with a flask containing 100 ml of medium. But, with Tween 85, this effect of aeration and agitation was not observed obviously (Fig. 3). This suggested that emulsification of hydrocarbons rather than the rate of aeration would act as one of the regulators of Candidal growth. Even though Tween 85 was added, however, growth of Candida sp. was inhibited slightly when above 150 ml of medium was used in a flask, because of inavailability of oxygen or low emulsification, as seen in Fig. 3.

We could not investigate the effect of aeration alone separately from emulsification, but these two factors are important and interesting to elucidate the kinetics of hydrocarbon fermentation.

3.4. Effect of inoculum size

Provided that the final cell concentration or net cell yield would be constant, the larger the inoculum size is, the more the fermentation period will be shortened. In this experiment, we attempted to determine the optimum inoculum size as small as possible without retardation of fermentation period and decrease of cell yield.

As shown in Table 2, when the inoculum size were 170-1020

mg/l, net cell yields were 3.4-4.0 g dry wt./l and the fermentation periods were 40 ± 5 hrs. Although the effect of inoculum size was not observed clearly, a reciprocal relationship was observed between the inoculum size and the lag phase of growth. This will become an important factor to produce yeast cell mass using a continuous process in industrial scale. From the result of Table 2, we decided the optimum inoculum size to be 200 mg dry cells/l.

Table 2. Effect of inoculum size on the fermentation period of C. albicans on hydrocarbon medium.

Incuvation time (hr)	Inoculum size (mg dry wt./l)				
	85	170	340	680	1020
	Cell yield (g dry wt./l)				
7.0	-	0.45	0.88	1.81	2.41
11.0	0.40	0.68	1.54	2.45	2.96
16.0	-	-	2.30	3.15	3.61
25.0	1.95	2.60	3.46	3.97	4.56
35.0	2.47	3.29	3.69	4.49	5.09
41.0	2.97	3.39	3.70	-	-
47.0	2.96	3.65	3.84	4.58	4.96

Cultivation was carried out on a rotary shaker (220 rpm) at 30°C.

3.5. Effects of concentration of carbon source and the ratio of carbon to nitrogen

It has been said that high concentration of water-soluble substrate inhibited microbial growth by influencing the osmotic pressure, while water-insoluble substrate such as hydrocarbon did not influence the growth rate. We expected that a high concentration of n-hexadecane would increase the interfacial area of hydrocarbon with yeast cells and subsequently enhance the cell yield and shorten the fermentation period. Concentration of n-hexadecane were varied from 0.5 to 4.0 %, and other constituents of the medium used were same as those in Table 1. The results shown in Table 3 indicate that the growth of cells is limited by other factors rather than the concentration of n-hexadecane, since the growth rate was not affected by the concentration of substrate up to 2.0 % but the assimilation percentage of substrate was greatly decreased from 89.4 to 13.5 %. When 4.0 % of substrate was used, decreased cell-yield and comparative long lag phase were observed. And so, we examined the effect of the carbon-nitrogen (C/N) ratio on the cell yield (Table 4). The result shows that it is necessary to maintain an adequate C/N ratio or constant pH.

Table 3. Effect of n-hexadecane concentration on Candidal growth

Concn. of n-hexadecane (v/v%)	C/N Ratio	Fermentation period(hr)	Cell yield (g dry wt./l)	Assimilation ratio (%)
0.5	3.8	26-33	2.94	89.4
1.0	7.5	41-49	3.86	58.7
2.0	15.0	41-49	4.06	30.8
4.0	30.1	49	3.65	13.5

Cultivation was carried out on a rotary shaker (220 rpm) at 30°C

Table 4. Effect of carbon/nitrogen ratio on the growth of C. albicans.

Concn. of NH_4NO_3 (g/l)	C/N ratio	Fermentation period(hr)	Cell yield (g dry wt./l)	Assimilation ratio (%)
1.0	37.6	49	3.16	48.0
2.5	15.0	49-57	3.76	57.1
5.0	7.5	49-57	4.15	63.1
10.0	3.8	49-70	4.75	72.1
15.0	2.5	70	4.73	71.9

Cultivation was carried out on a rotary shaker (220 rpm) at 30°C. Concentration of n-hexadecane was 1.0 v/v%.

3.6. Effect of pH on growth rate of Candida albicans

As mentioned above, the growth rate of the yeast was affected by the concentration of NH_4NO_3, particularly of NH_4^+ ion, or by pH of culture broth. When Candida albicans was cultured on the medium shown in Table 1, pH of broth slightly increased at the lag phase of growth, and then dropped suddenly to 2.1-2.2. At this pH, yeast growth was inhibited and substrate could not be assimilated completely. pH of broth dropped according as the consumption of ammonium ion, and so pH of broth was maintained constantly at 5.2-5.4 by adding NH_4OH. As shown in Fig. 4, growth rate and cell yield increased fairly by addition of NH_4OH. Although the growth rate diminished temporarily immediately after the addition of NH_4OH, soon after this lag phase cells grew exponentially until pH of broth dropped to 2.2 (Fig. 5). In this manner we obtained the assimilation ratio of 80.5 % at the concentration of 1 % n-hexadecane, whereas no addition of NH_4OH, the assimilation ratio was 56.1 %.

4. Discussion

Johnson suggested that oxygen supply was a rate limiting factor on the growth of microorganisms in hydrocarbon fermentation[1]. On the contrary, Blakebrough suggested that

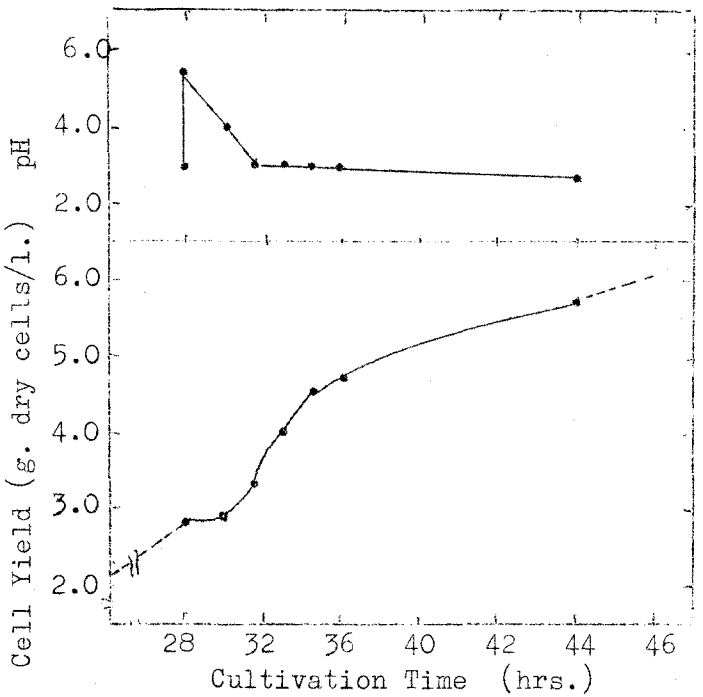


Fig. 5. Effect of NH_4OH on the cell propagation. pH was adjusted at 28 hr of cultivation.

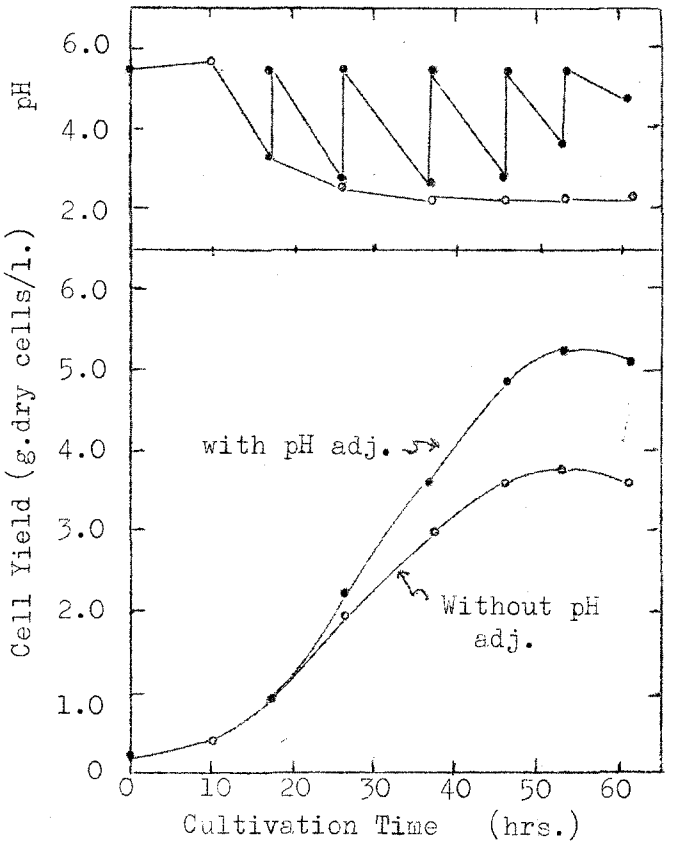


Fig.4. Effect of pH adjustment on cell yield. Cultivation was carried out on a rotary shaker (220 rpm) at 30°C.
● -● ; with pH adjustment,
o -o ; without pH adjustment.

emulsification of hydrocarbons rather than oxygen supply was rate-limiting using several fermentation equipment[2].

But reduced aeration below critical demand of oxygen has influences on the growth rate of Candida albicans. Johnson[1] also suggested that the growth rate was severely influenced by oxygen-supply during the later part of the growth period because of reduction of dissolved oxygen. We observed that pH of the medium was rather limiting during this period of growth when a detergent was added[3]. Yeast could grow a relatively low pH compared with bacteria, but at an extremely low pH region the growth activity of the yeast was reduced and then growth rate would be lowered. And by addition of NH_4^+ ion, the growth rate was maintained constantly with the same oxygen-supply. We concluded that emulsification of hydrocarbons and maintenance of constant pH were important limiting-factors for Candidal growth on hydrocarbons.

5. Summary

During the course of the studies on the ability of hydrocarbon utilization and cytochrome c production of yeast, we found out that Candida albicans was useful for these purposes. Then, we investigated the effects of medium

constituents, pH of culture medium, and aeration on cell yield of Candida albicans in this chapter.

Addition of corn steep liquor and a detergent, Tween 85, shortened the period of fermentation. Maintenance of pH at 5.2-5.4 increased the cell yield. The effect of inoculum size was not obvious. The effect of aeration was observed in the absence of the detergent, but not in the presence of the detergent, under the conditions tested.

6. References

1). M.J. Johnson: Chem. Ind., 36, 1532 (1964)

2). N. Blakebrough, P.G. Shepherd and I. Nimmons: Biotech. Bioeng., 9, 77 (1967)

3). A. Tanaka, H. Maki and S. Fukui: J. Ferment. Technol., 45, 1156 (1967)

Chapter 2. Production of cytochrome c by Candida albicans

1. Introduction

It has been observed that oxidative degradation of hydrocarbons by microorganisms is carried out under more aerobic conditions than that of carbohydrate[1]. Hence our attention was called to the question whether the yeasts grown on hydrocarbons as a sole carbon source would produce a large amount of respiratory enzymes, especially cytochrome c. In this study we investigated the ability of hydrocarbon utilization and cytochrome c productivity of several kinds of yeasts, and found out that Candida albicans was suitable for both purposes.

Oxygen-uptake of Candida albicans was inhibited by addition of sodium azide, and this suggested that the respiration of this yeast would proceed via an electron transport system including at least cytochromes a_1 and a_3. Also, we could detect cytochromes a, b, and c in reduced intact cells spectrophotometrically by the opal-glass method of Shibata[2].

We also attempted to study on the relation among growth rate, cytochrome c content, and respiratory activity, and to use the cytochrome c content as an index of growth activity

of cells. The results obtained showed that the growth activity of the yeast correlated apparently with the cytochrome c content.

Under the cultural conditions used, we obtained a maximal cytochrome c content of 1.5 mg per g dry cells of Candida albicans.

2. Experimental procedure

2.1. Microorganism

The organism mainly used in this study was Candida albicans Berkhout IFO 0583.

2.2. Determination of cytochrome c content

2.2.1. Extraction of cytochrome c with CTAB (cetyltrimethylammonium bromide)

Cytochrome c of yeast was extracted with CTAB according to the method of Ohbayashi[3]. Intact cell suspension (containing 50-60 mg dry cells/ml) was adjusted to pH 3.5 with 1 N acetic acid, added with CTAB (50-60 μg/mg dry cells), kept at 4°C for 45 min, and centrifuged. Sedimented cells were resuspended to the same volume of 0.05 M of phosphate buffer, pH 8.3, kept at 37°C for 4 hrs, and then centrifuged. Super-

natant obtained by this treatment contained cytochrome c.

2.2.2. Estimation of cytochrome c

Reduction of cytochrome c in the supernatant was performed by addition of a small amount of $Na_2S_2O_4$ to the supernatant obtained above. Content of cytochrome c was estimated from Equation 1[4] by measuring the optical density at 550 mμ (absorption maximum of α-absorption band of reduced cytochrome c) and 535 mμ (absorption minimum) using Shimadzu Spectrophotometer type QB-50.

2.2.3. Determination of cytochrome c content of intact cells

Cytochrome c in cells was reduced by addition of excess of $Na_2S_2O_4$ to the intact cell suspension containing about 30 mg dry cells/ ml. Spectra of the cytochromes contained in the intact cells were measured by the opal-glass method using Shimadzu Multipurpose-spectrophotometer. Content of cytochrome c was calculated from Equation 1[4] by reading the optical density at 550 mμ (absorption maximum) and 535 mμ (absorption minimum), respectively.

$$C(\text{moles of cytochrome c/cm}^3) = \frac{D_{550}-D_{535}}{E_{550}-E_{535}} \times \frac{1}{L}$$

(Equation 1)

$$C^*(\mu\text{g. of cytochrome c/cm}^3) = 600 \times (D_{550} - D_{535}).$$

where,

E_{550} = 2.77 X $10^7 cm^2/mol$,

E_{535} = 0.77 X $10^7 cm^2/mole$,

D_{550} = optical density at 550 mμ,

D_{535} = optical density at 535 mμ,

L = light path (1cm),

molecular weight of cytochrome c = 12,000.

2.3. Measurement of oxygen-uptake

Oxygen-uptake of the cells was measured by a conventional Warburg manometric technique, the final total volume in each vessel being 3.2 ml, made up as follows; 1.0 ml of cell suspension containing about 5 mg dry cells, 1.0 ml of 0.033 M KH_2PO_4, and distilled water in the main chamber, 0.01 mmole glucose or 0.16 mmole n-hexadecane as substrate in the side arm, and 0.2 ml of 20 % (W/V) KOH and filter paper in the center well. In the case of the inhibitory experiment, 0.03 mole of NaN_3 was added into the main chamber. After preincubation for 10 min, measurement of oxygen-uptake was begun.

3. Results

3.1. Ability of growth on n-hexadecane and cytochrome c productivity of several kinds of yeast

In order to screen the yeast that grows well on hydrocarbon and produces a large amount of cytochrome c, we examined several strains of yeast for their ability to grow on n-hexadecane and their productivity of cytochrome c on glucose medium shown in Table 2 in Part I. Growing on glucose medium, Candida albicans and Pichia membranaefaciens produced a large amount of cytochrome c as shown in Table 1. While, Candida albicans, Candida lipolytica, Candida tropicalis, and YH 101C1 strain (isolated from garden soil) grew well on n-hexadecane, but Pichia membranaefaciens did not (Table 1). From this result, we used Candida albicans in the subsequent experiments.

Table 1. Hydrocarbon utilization and cytochrome c production by yeast.

Yeast	Growth on n-hexadecane	Cytochrome c production in glucose medium mg/g dry cells
Candida albicans Berkhout IFO 0583	++	1.21
Candida lipolytica IFO 0717	+	0.13
Candida utilis IFO 0619	-	0.44
Candida tropicalis IFO 0589	++	0.44
Debaryomyces kloeckeri IFO 0034	-	0.09
Hansenula anomala IFO 0127	-	0.64
Pichia membranaefaciens IFO 0188	-	1.85
Saccharomyces sake No.7	-	0.24
YH101-C1(isolated from soil)	++	0.52

Cultivation was carried out on a rotary shaker (220 rpm) at 30°C for 7 days on n-hexadecane or for 24 hr on glucose.
Cytochrome c content of intact cells was measured using Shimadzu Multipurpose-spectrophotometer by the opal-glass method of Shibata[2].
++: heavy growth, +: moderate growth, -: no growth.

3.2. Absorption spectrum of cytochromes produced by Candida albicans

α-Absorption bands of reduced cytochromes a, b, and c were detected at 600, 560, and 550 mμ, respectively, in the cells of Candida albicans grown on both glucose and n-hexadecane (Fig. 1). These spectra were measured using intact cell suspension containing $Na_2S_2O_4$, and were same as those of CTAB extract, especially with respect to cytochrome c.

We did not purify and characterize cytochrome c produced by Candida albicans in this study. But, based on the results of purification and characterization of cytochrome c produced by Saccharomyces sake[5], we confirmed that the absorption spectrum shown in Fig. 1 represented that of reduced cytochrome c. Therefore, we used these absorption spectra for the estimation of cytochrome c content of intact cells.

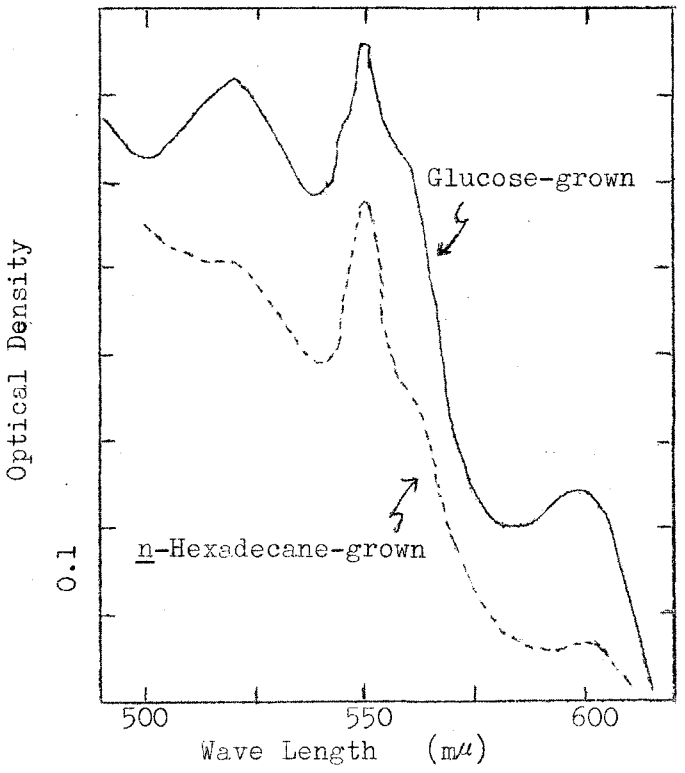


Fig. 1. Absorption spectrum of reduced cytochromes as measured with intact Candidal cells using Shimadzu Multipurpose-spectrophotometer by the opal-glass method of Shibata[2].

—— ; glucose-grown cells

... ; n-hexadecane-grown cells

3.3. Effects of medium constituents and cultural conditions on the production of cytochrome c by Candida albicans

3.3.1. Effect of a detergent, Tween 85

As demonstrated in the preceding chapter, addition of Tween 85 shortened the cultivation period of this yeast but did not affect the final cell yield. Cytochrome c production

was also stimulated by addition of the detergent as shown in Table 2. A maximal specific content of cytochrome c was obtained during the middle or the later part of the exponential growth phase as we expected. When 200 μl/l of Tween 85 was added, we obtained 1.47 mg per g dry cells or 7.50 mg per l of broth, of cytochrome c.

Table 2. Effect of Tween 85 on cytochrome c production by C. albicans.

Cultivation time (hr)	Amount of Tween 85 added (μl/l)							
	0	10	100	200	0	10	100	200
	cytochrome c produced (mg/g dry cells)				(mg/l)			
21.5	-	-	0.76	1.00	-	-	1.19	1.12
45.0	-	0.98	0.95	1.47	-	2.70	4.32	7.50
69.0	-	0.81	0.81	0.67	-	3.16	3.00	3.15
93.5	0.17	0.43	0.43	0.40	0.09	1.91	1.51	1.56
118.0	0.86	0.22	0.22	-	1.81	0.71	0.79	-
142.0	0.77	-	-	-	3.37	-	-	-
166.0	0.68	-	-	-	2.99	-	-	-

Cultivation was carried out on a rotary shaker (220 rpm) at 30°C. Cytochrome c content of intact cells was measured using Shimadzu Multipurpose-spectrophotometer.

3.3.2. Effect of aeration

In the presence of a detergent, aeration or agitation did not exert any significant effect on the growth rate, final cell yield, and cytochrome c production of Candida albicans in the n-hexadecane medium. Varying the volumes of medium in 500-ml shaking flasks from 25 to 150 ml, we examined the

effect of aeration or agitation on Candidal production of cytochrome c, and obtained from 1.3 to 1.5 mg per g dry cells of cytochrome c in any cases (Table 3).

Table 3. Effect of aeration on cytochrome c production by C.albicans.

Cultivation time (hr)	Volume of medium in a 500 ml -shaking flask(ml)									
	25	50	75	100	150	25	50	75	100	150
	Cytochrome c produced.									
	(mg/g dry cells)					total (mg/l)				
26.5	1.31	0.99	1.16	1.47	0.84	2.33	1.72	1.91	2.32	1.28
35.0	1.15	1.31	1.51	1.33	1.34	3.29	3.60	4.11	3.64	3.14
45.0	1.23	1.42	1.49	1.46	1.23	3.33	4.22	4.41	4.61	3.17
54.0	0.72	0.84	0.88	0.83	0.91	2.59	2.69	3.08	2.83	2.84
66.0	0.43	0.51	0.57	0.58	0.69	1.51	1.82	1.92	2.09	2.25

Method of cultivation and cytochrome c determination were same as those shown in Table 2.

3.3.3. Effect of inoculum size

By increasing the inoculum size, we tried to yield a larger amount of cytochrome c in a shorter cultivation period, since the larger the inoculum size was, the more the lag phase of growth was shortened as mentioned in the previous chapter. But the attempt, so far tested, was not successful.

3.3.4. Effect of pH adjustment of medium

During the course of the fermentation, pH of the broth was lowered according as the consumption of NH_4^+ ion and the release of NO_3^- ion. This gave bad effects

on the growth rate and cell yield of this yeast. With pH adjustment at intervals, the yeast growth was stimulated, and the cell yield and cytochrome c production were enhanced as shown in Table 4. This suggested that continuous pH adjustment and addition of substrate at intervals might produce a large amount of cell mass and cytochrome c. Surely, we could produce a larger amount of cell mass in this manner, but did not determine the content of cytochrome c.

Table 4. Effect of pH adjustment on cytochrome c production by C. albicans.

Cultivation time (hr)	Without pH adjustment			With pH adjustment		
	cell yield (g/l)	cytochrome c produced (mg/g cells)	(mg/l)	cell yield (g/l)	cytochrome c produced (mg/g cells)	(mg/l)
17	0.93	-	-	0.93	-	-
26	1.94	0.39	0.77	2.22	1.45	3.22
37	2.98	0.97	2.89	3.60	0.93	3.35
48	3.55	0.81	2.88	4.87	0.99	4.82
53	3.70	0.70	2.59	5.25	0.85	4.46
61	3.59	0.70	2.51	5.10	0.92	4.69

pH of medium was adjusted with 2% NH_4OH. Others were same as those shown in Table 2.

3.3.5. Effects of precursors for cytochrome c synthesis

In order to obtain the larger amount of cytochrome c in hydrocarbon fermentation, the effects of various precursors for cytochrome c synthesis were investigated using the "hydrocarbon mixture 2" (rich in n-undecane) as a sole carbon source. The additives used were ferric chloride for heme

synthesis, glycine and succinate for synthesis of porphyrin skeleton, and amino acid mixture (casamino acid) for protein synthesis. But these attempts were all failed in any cases.

3.4. Oxygen-uptake by Candida albicans grown on glucose and n-hexadecane

In order to elucidate whether Candida albicans respires via cytochrome system or not, and that the enzyme system participating hydrocarbon oxidation is constitutive or inducible, we compared the oxygen-uptake of cells grown on n-hexadecane with that of cells grown on glucose. Cells grown on glucose showed higher oxygen-uptake than cells grown on n-hexadecane, or on both substrates, glucose and n-hexadecane. This suggested that hydrocarbon oxidizing enzymes would be constitutive rather than inducible. This was consistent with the fact that Candida albicans preincuvated on glucose and on n-hexadecane respectively, exhibited the same growth rate when transferred into n-hexadecane medium.

Addition of sodium azide inhibited the respiration of Candida albicans using either glucose or n-hexadecane as substrate. From this result and that shown in Fig. 1, we supposed that Candida albicans would respire via cytochrome system, at least cytochrome a. We could not find out a

significant difference between the glucose-grown and the n-hexadecane-grown cells concerning their growth activity and respiratory activity on n-hexadecane.

3.5. Relations between growth rate, cytochrome c content, and oxygen-uptake of *Candida albicans* growing on n-hexadecane

As mentioned above, the cytochrome c content of the yeast reached at the maximum during the exponential growth phase. On the contrary, respiratory activity became maximum at the end of the lag phase or at the beginning of the exponential phase of the growth. Typical results were shown in Fig. 2 and 3. Initially, we attempted to use the cytochrome c content or respiratory activity as an index of the growth activity of the yeast, and found out that the cytochrome c content would be able to use for this index sufficiently. As shown in Fig. 4, the specific growth rate at the middle or the end of the exponential growth phase was parallel with the cytochrome c content of the yeast cells. The cytochrome c content of the intact cells can be determined quickly using a Shimadzu Multipurpose-spectrophotometer by the opal-glass method[2], and therefore, will be a excellent method to control the growth activity of cells in a fermentation process.

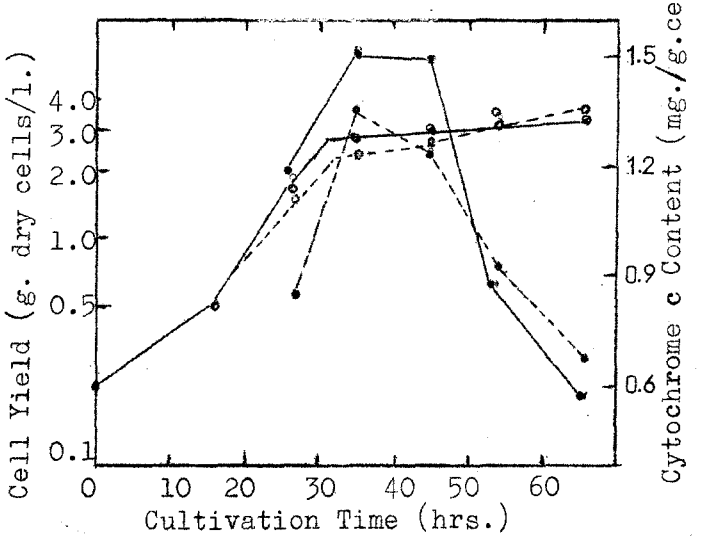


Fig.3. Relation between the yeast growth and the cytochrome c content.
Cultural condition and measurement of cytochrome c were same as those in Table 3.
——; run 1, (○; cell yield and ●; cytochrome c content)
...; run 2, (○; cell yield and ●; cytochrome c content)

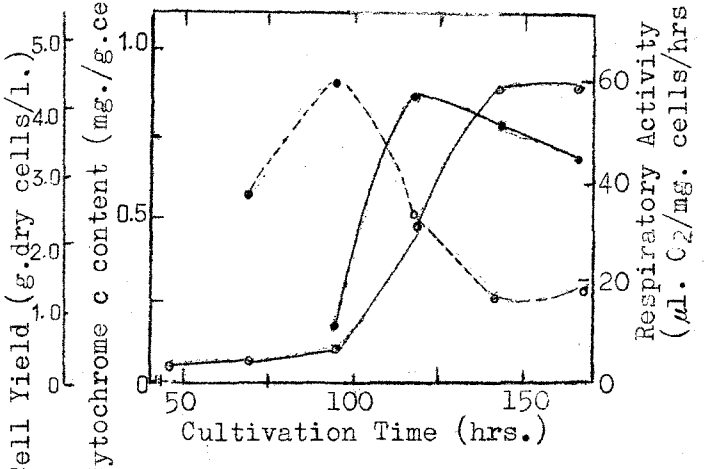


Fig.2. Relations among the Candidal growth, the respiratory activity, and the cytochrome c production.
Cultivation medium contained no detergent.
Others were same as those in Table 3.
○—○; cell yield
●—●; cytochrome c content
○··○; respiratory activity, expressed by oxygen-uptake of harvested cells

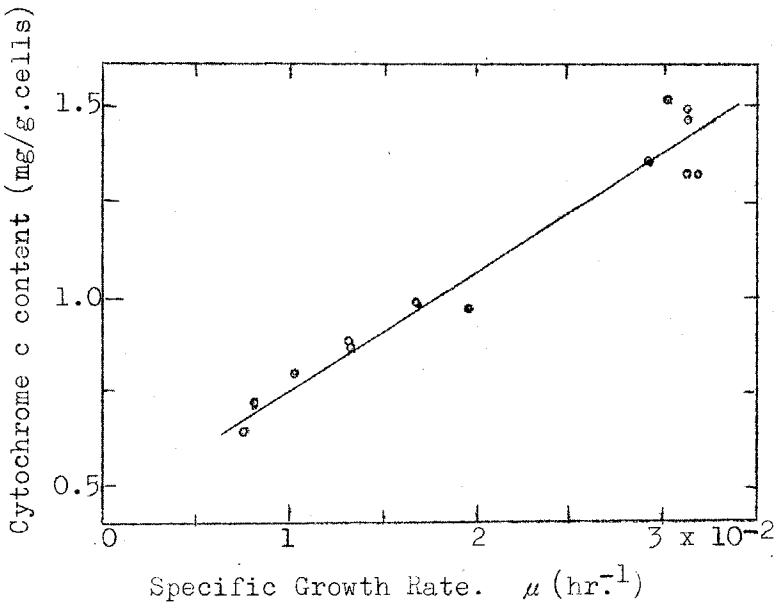


Fig. 4. Relation between the specific growth rate and the cytochrome c content of Candida albicans.
These were measured at the middle or at the end of exponential growth phase. Cultural condition and measurement of cytochrome c content were same as those in Table 3.

4. Discussion

Initially, we attempted to produce cytochrome c from yeast, and screened several strains of yeast for their ability to grow on hydrocarbon and to produce cytochrome c. We found out that Candida albicans was suitable for both purposes. Using this yeast, we examined the effects of the medium constituents and the cultural conditions, and found out that addition of Tween 85 and adjustment of pH greatly stimulated cytochrome c production of Candida albicans, but that inoculum size, aeration, and addition of the precursors for cytochrome biosynthesis did not have any significant effect. When added 200 μl per liter of Tween 85, we obtained 7.50 mg per liter or 1.47 mg per g dry cells, of cytochrome c. This figure was comparable to that of cells grown on glucose as shown in Table 1. Addition of detergent and adjustment of pH increased the growth rate, and consequently stimulated cytochrome c production, since the growth rate, especially at the middle or the end of exponential growth phase, was related to cytochrome c production of this yeast as shown in Fig. 4. Thus, we obtained 1.5 mg per g dry cells of cytochrome c at the maximum. But this yield was not so large as we expected in the hydrocarbon fermentation.

Secondary, we attempted to ascertain that the respiration

of Candida albicans would proceed via cytochrome system when grown on hydrocarbon. We first compared the absorption spectrum of intact Candidal cells grown on n-hexadecane with those of this yeast and Saccharomyces sake[5] grown on glucose, and confirmed that Candida albicans grown on n-hexadecane contained cytochrome a, b, and c (Fig. 1). Next, we investigated the decrease of oxygen-uptake using the yeast cells grown on glucose and n-hexadecane. Oxygen-uptake of both Candidal cells was inhibited by addition of sodium azide, and we concluded that Candida albicans would respire via cytochrome system as other yeasts did.

Third, we examined the relations among the growth rate (or the specific growth rate), oxygen-uptake, and cytochrome c content of the yeast cells grown on n-hexadecane. As shown in Fig. 2, 3, and 4, the growth rate at the later part of the exponential phase was closely related with the cytochrom c content. It was observed that the cytochrome c content was small during the induction period of the growth and began to increase concomitantly with the start of the exponential phase when the oxygen-uptake of the cells became its maximum. The increase of the cytochrome c content proceeded in parallel with the yeast growth during the exponential phase and reached to the maximum at the later stage of the exponential phase

when the specific growth rate decreased suddenly, afterward the content falled off gradually. Subsequently, we will have to harvest the yeast cells at the maximal step in order to obtain cytochrome c from the cells. The facts mentioned above suggest that the cytochrome c content can be used as an index of the growth activity of this yeast in a hydrocarbon fermentation process.

5. Summary

This chapter dealt with the study on the relations among the growth rate, cytochrome c content and respiratory activity of Candida albicans when the yeast was cultivated on n-hexadecane under aeration.

Under the cultural conditions employed, the maximal cytochrome c content was about 1.5 mg per g dry cells, which was analogous to that of glucose-grown cells. The growth rate was closely related with the cytochrome c content. The latter increased in parallel with the yeast growth, reached to the maximal value at the later part of the exponential phase, then falled off gradually. The correlationship between the cytochrome c content and the specific growth rate showed that the former can be used as an index of the growth activity of the yeast.

6. References

1). M.J. Johnson: Chem. Ind., 1964, 1532

W.A. Darlington: Biotech. Bioeng., 6, 241 (1964)

2). K. Shibata: J. Biochem., 45, 599 (1958)

K. Shibata: Method of Biochem. Analysis, Vol. VII, p. 77 (Interscience Publishers, Inc., New York) (1959)

3). A. Ohbayashi: Amino acid. Nucleic acid, 11, 31 (1965)

4). O. Rosenthal: J. Biol. Chem., 148, 431 (1943)

5). S. Ueno, K. Sumino, Y. Tani and S. Fukui: Nogei-Kagaku Kai-Shi, 42, 139 (1968)

Chapter 3. Production of cytochrome c by various yeasts

1. Introduction

As mentioned in Chapter 2, although Candida albicans produced relatively large amount of cytochrome c in hydrocarbon fermentation among the test organisms used, the content obtained was far smaller than expected. In order to obtain larger amount of cytochrome c from hydrocarbons, we investigated the cytochrome c productivity of hydrocarbon-utilizing yeasts obtained from several institutes and from natural sources. Utilizing the "hydrocarbon mixture 2" (rich in n-undecane), Candida lipolytica NRRL y-6975 showed the highest cytochrome c productivity, although the new isolates from garden soil also yielded high cytochrome c contents.

2. Experimental procedure

2.1. Cultivation method and estimation of cytochrome c

Cultivation of yeasts and estimation of cytochrome c were same as those shown in Chapter 1 and 2 of this part except that the "hydrocarbon mixture 2" (n-decane 22.2, n-undecane 44.5, n-dodecane 29.1 and n-tridecane 4.2 % by weight) as a carbon source and $NH_4H_2PO_4$ as a nitrogen source

were used.

2.2. Isolation of yeast from natural sources

Hydrocarbon-utilizing yeasts were isolated from garden soil by enrichment culture technique[1,2]. That is, a soil sample was incubated at 30°C on a rotary shaker (220 rpm) for 2 days in a medium consisting of NH_4NO_3 5.0 g, KH_2PO_4 2.5 g, $MgSO_4 \cdot 7H_2O$ 1.0 g, $FeCl_3 \cdot 6H_2O$ 0.02 g, chloramphenicol 0.02 g, tap water 1000 ml, hydrocarbon 10-20 ml (n-hexadecane, "hydrocarbon mixture 2" or light oil), and pH 5.2. One mililiter of the cultured broth was transferred into 100 ml of the same medium and was further incubated for 2 days under the same condition. The resulting cells were diluted with sterilized saline to 20-30 cells per ml, and were cultured on malt-agar plates for 3 days. Colonies of yeast thus obtained were cultured again in the liquid hydrocarbon medium, and on the malt-agar plate, successively. Same procedure was repeated until pure cultures were obtained. The purity of the isolated yeasts was confirmed microscopically. The yeasts isolated were maintained on malt-agar slants. By this method, yeasts and molds could be isolated.

3. Results and discussion

3.1. Cytochrome c production by hydrocarbon-utilizing yeasts

As shown in Table 1, Candida lipolytica NRRL y-6795 and several new isolates produced large amount of cytochrome c using the "hydrocarbon mixture 2" as a sole carbon source. Candida albicans, however, showed the relatively low content on this substrate. Among these yeasts, Candida lipolytica NRRL was superior in the cytochrome c productivity, then we investigated the effects of some medium constituents on the cytochrome c production by this yeast.

3.2. Production of cytochrome c by Candida lipolytica NRRL

3.2.1. Effect of ferric ion

Although the effect of ferric ion was seen on the yeast growth, the cytochrome c productivity was not affected even under the minimum addition of ferric chloride as shown in Table 2. The cytochrome c content of yeast cells was rather smaller at the high concentration of ferric ion.

3.2.2. Effects of precursors

δ-Aminolevulinic acid, a precursor for heme synthesis, is known to be formed from glycine and succinate under the

Table 1. Cytochrome c production by yeasts on hydrocarbon

Organism	Cell yield g dry cells/l	Cytochrome c produced mg/l	Cytochrome c produced mg/g cells
Candida albicans IFO 0583	6.29	3.71	0.59
Candida intermedia NRRL Y-6328-1	4.01	5.26	1.31
Candida lipolytica IFO 0717	6.42	6.70	1.05
Candida lipolytica NRRL y-6795	7.01	10.33	1.47
Candida tropicalis IFO 0589	4.28	2.59	0.61
Candida tropicalis pK-233	3.38	3.36	0.99
YH 101C1	7.35	7.06	0.96
YL 102B1	4.96	8.42	1.70
YL 102B2	5.09	8.28	1.63
YL 103A2	5.91	9.04	1.53
YL 201A1	5.00	9.04	1.81
YL 205A3	5.02	8.10	1.61
YM 103A3	4.91	7.60	1.55
YM 105A2	5.39	9.54	1.77
YM 105C1	4.92	7.88	1.60
YM 105C3	4.79	8.75	1.83
YM 105D1	4.75	7.38	1.55
YM 201A1	4.78	7.67	1.60
YM 204A2	5.96	8.35	1.40

The yeasts were cultured on the "hydrocarbon mixture 2" for 42 to 45 hrs with shaking.

participation of δ-aminolevulinic acid synthetase. Therefore, we expected that the addition of glycine and succinate would stimulate the cytochrome c production by this yeast. But this attempt was failed as seen in Table 3.

3.2.3. Effects of vitamins

The synthesis of coproporphyrin and hemin by Saccharomyces anamensis is known to be affected seriously by some vitamins[3]. Herefrom we studied on the effect of some water-soluble vitamins on the cytochrome c production by this yeast. As shown in Table 4, no effect was observed concerning to the yeast growth and cytochrome c productivity.

4. References

1). K. Yamada, S. Horiguchi and J. Takahashi: Agr. Biol. Chem., 29, 943 (1965)

2). A. Tanaka and S. Fukui: Mizu-Shori Gijutsu, 8 (10), 9 (1967)

3). W. Stich and H. Eisgrober: Z. Physiol. Chem., 287, 19 (1951)

Table 2. Effect of ferric ion on production of cytochrome c by Candida lipolytica NRRL

$FeCl_3 \cdot 6H_2O$ mg/l	Cell yield g cell/l	Cytochrome c produced mg/l	mg/g cell
2	5.98	12.24	2.05
5	6.09	11.81	1.94
10	6.30	11.66	1.84
20	6.43	11.09	1.72
50	6.38	10.91	1.71
100	6.41	10.91	1.70

The yeast was cultured for 44 hrs.

Table 3. Effects of glycine and succinate on cytochrome c production by Candida lipolytica NRRL

Glycine mmole/l	Succinate mmole/l	Cell yield g cell/l	Cytochrome c produced mg/l	mg/g cell
0	0	6.45	11.66	1.81
0.1	0	6.12	9.79	1.60
1.0	0	6.07	11.09	1.83
0	0.1	6.15	10.87	1.77
0	1.0	6.40	10.48	1.63
0.1	0.1	6.61	11.09	1.68
1.0	1.0	6.43	10.71	1.67

The yeast was cultured for 44 hrs on a rotary shaker (220 rpm).

Table 4. Effect of vitamins on cytochrome c production by Candida lipolytica NRRL

Vitamin	Amount added mg/l	Cell yield g cell/l	Cytochrome c produced mg/l	mg/g cell
None	---	7.21	9.96	1.38
Vit. B_1	1.0	7.08	10.51	1.48
Vit. B_2	0.2	7.20	10.08	1.40
Vit. B_6	1.0	7.20	9.94	1.38
Vit. B_{12}	0.1	7.70	9.79	1.27
Biotin	0.1	7.20	10.13	1.41
Folic acid	0.2	7.09	9.79	1.38
p-Aminobenzoic acid	1.0	7.26	9.98	1.37
Ca-pantothenate	1.0	7.18	9.50	1.32
Inosit	5.0	7.21	9.79	1.36
Nicotinic acid	1.0	7.09	9.32	1.31
Nicotinamide	1.0	7.00	9.41	1.34

The yeast was cultured for 45 hrs.

Part III. Production of carotenoids by *Mycobacterium smegmatis* in hydrocarbon media

Chapter 1. Studies on the cultural conditions

1. Introduction

In the course of the studies on the ability of vitamin B_{12}-producing bacteria to utilize hydrocarbons, we observed that some bacteria including *Mycobacterium smegmatis*, *Nocardia lutea* and *Nocardia corallina* grown on *n*-hexadecane, made up a clump of cells occluding *n*-hexadecane and produced orange to orange-red pigments. These pigments were partly extracted into hydrocabon layer from cells during the cultivation and were easily extractable with *n*-hexane or *n*-hexane-acetone from cells. The pigments produced by *Mycobacterium smegmatis* mainly investigated on their column chromatographic behaviors and some chemical characteristics, were identified as carotenoids composed of at least six components, some of which were derivatives of γ-carotene. The chemical structures of these carotenoids will be described in the following chapter. Although these carotenoids will not be useful as provitamin A, they are appliable for food-coloring agents. In this chapter, we investigated the optimal cultural condition to

produce a large amount of carotenoids by hydrocarbon fermentation.

2. Experimental procedure

2.1. Microorganism

The organism used in this study was Mycobacterium smegmatis IFO 3080. This organism was maintained on conventional natural nutrient-agar slant.

2.2. Cultivation method

For an inoculum, the bacterium was cultured on conventional nutrient-agar slant for 48 hrs at 30°C. A loopful of the cells was transferred into 100 ml of glucose medium shown in Table 1 in a 500-ml shaking flask, which was incubated at 30°C on a rotary shaker (220 rpm) for 72 hrs. After washing twice with sterilized saline, cells were suspended in 50 ml of saline, and 3 ml of the cell suspension was added to 100 ml of hydrocarbon medium shown in Table 1, in a 500-ml shaking flask; the culture was incubated at 30°C on a rotary shaker (220 rpm).

2.3. Estimation of carotenoids produced

The crude mixture of carotenoids produced by Mycobacterium

smegmatis had an absorption maximum at 470 mμ in petroleum ether solution as shown in Fig. 1. Although the accurate absorption coefficient of each of the crude carotenoids was not determined, $E^{1\%}_{1\ cm}$ of β-carotene, 2580,[1] was temporarily used as a standard for the estimation of their total contents.

Extraction of carotenoids from cells was carried out as follows; cells were separated by centrifugation and (or) filtration from culture medium, and extracted with acetone-petroleum ether (2:3) for 24 hrs in the dark at room temperature, and then resultant extract was washed with water until no more acetone remained in the petroleum ether layer. An optical density of this extract was measured at 470 mμ after diluted to an appropriate volume with petroleum ether, then its carotenoid content was estimated as mentioned above.

2.4. Determination of cell yield

The carotenoid-extracted cell residues were weighed after being dried at 110°C for 6 hrs.

2.5. Hydrocarbons

Pure n-alkanes were obtained commercially. "Hydrocarbon mixture 1 and 2" were obtained from Maruzen Petroleum Industries Co. and "hydrocarbon mixture 3" was an equal volume mixture of

Table 1. Composition of basal medium used.

Carbon source*	
$(NH_4)_2SO_4$	1.5 g
$(NH_2)_2CO$	0.5 g
KH_2PO_4	2.0 g
$Na_2HPO_4 \cdot 12H_2O$	3.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$CaCl_2 \cdot 2H_2O$	0.01 g
$FeSO_4 \cdot 7H_2O$	0.005 g
$MnSO_4 \cdot nH_2O$	0.005 g
$CoSO_4 \cdot 7H_2O$	0.001 g
Tap water	1000 ml
pH 7.0	

*Carbon source: Hydrocarbon medium, hydrocarbon 20 ml.
Glucose medium, glucose 32.9 g

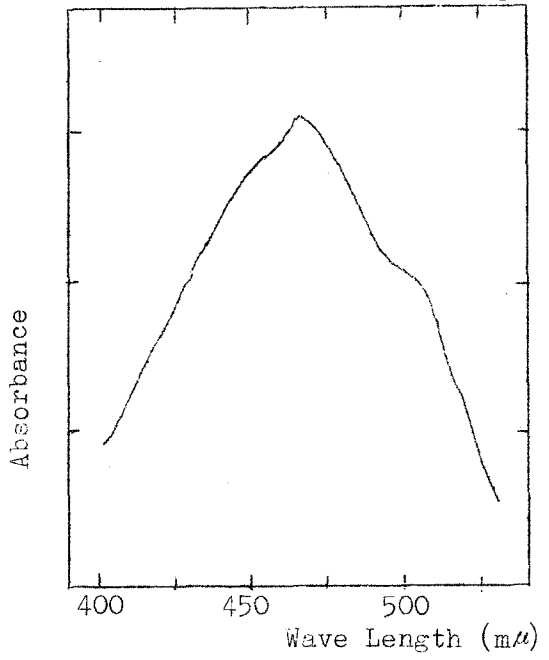


Fig.1. Absorption spectrum of crude carotenoids extracted from M. smegmatis cells (in n-hexane).

"hydrocarbon mixture 1 and 2". n-Alkane compositions of these mixtures were shown in Table 2.

Table 2. n-Alkane compositions of the "hydrocarbon mixtures 1, 2, and 3".

	"Mixture 1"	"Mixture 2"	"Mixture 3"
		% by weight	
n-Decane (C_{10})	...	22.2	11.1
n-Undecane (C_{11})	2.3	44.5	23.4
n-Dodecane (C_{12})	13.4	29.1	21.2
n-Tridecane (C_{13})	51.3	4.2	27.8
n-Tetradecane(C_{14})	33.0	...	16.5

3. Results

3.1. Effect of various carbon sources on carotenoid production

As shown in Table 3, Mycobacterium smegmatis could assimilate n-alkanes of C_{11-19}, especially n-hexadecane, and their mixtures as well. This bacterium did not grow on n-eicosane till 6th day but a longer cultivation permitted the bacterial growth by assimilation of the alkane. As for the carotenoid production, n-hexadecane and n-tetradecane were superior, while hydrocarbon mixtures also enabled the production of a relatively large amount of carotenoids. From these results, we used n-hexadecane and "hydrocarbon mixture 2 and 3" (Table 2) as carbon sources in the subsequent experiments.

Table 3. Effect of various hydrocarbons on the production of carotenoids by M. smegmatis.

Carbon source	Cell yield (g-dry cells/l)	Carotenoid produced (μg/l)	Carotenoid produced (μg/g cells)
n-Hexane	no growth	-	-
n-Heptane	no growth	-	-
n-Octane	no growth	-	-
n-Nonane	no growth	-	-
n-Decane	no growth	-	-
n-Undecane	0.99	118	119.1
n-Dodecane	2.00	80	40.0
n-Tridecane	3.21	200	62.4
n-Tetradecane	3.21	305	95.0
n-Pentadecane	2.60	160	61.5
n-Hexadecane	3.74	340	91.7
n-Heptadecane	3.11	167	53.8
n-Octadecane	3.20	180	56.3
n-Nonadecane	3.65	121	33.2
n-Eicosane	no growth	-	-
"Mixture 1"(C_{11-14})	2.48	238	95.9
"Mixture 2 (C_{10-13})	2.95	288	97.6
"Mixture 3"(C_{10-14})	2.95	284	96.3

Cultivation was carried out on a rotary shaker (220 rpm) at 30°C for 6 days in basal medium shown in Table 1 except carbon source.

3.2. Effect of natural nutrients

Since the composition of the medium used in these studies was very simple, it was expected that certain organic substance would have a good effect on the bacterial growth and carotenoid production. As shown in Table 4, 0.05 % of fish meat extract stimulated the carotenoid synthesis slightly, but other natural nutrients, so far tested, did not show any effect on the carotenoid production by Mycobacterium smegmatis, although these stimulated bacterial growth.

Table 4. Effect of natural nutrients on the carotenoid production.

Addition	Amount added (mg/l)	Cell yield (g-dry cells/l)	Carotenoid produced (μg/l)	Carotenoid produced (μg/g cells)
Fish meat extr.	500	3.31	459	138
	100	3.30	290	88
Corn steep liquor	500	3.55	324	91
	100	3.13	252	77
Yeast extr.	500	3.61	370	102
	100	3.29	348	106
Casamino acid	500	3.55	320	90
	100	3.09	294	95
Peptone	500	3.69	348	94
	100	3.17	304	96
Malt extr.	500	3.17	262	83
	100	3.41	264	77
None	-	3.02	336	111

Cultivation was carried on a rotary shaker (220 rpm) for 6 days using the "hydrocarbon mixture 2" as a carbon source.

3.3. Effect of amino acids

Fish meat extract showed some promoting effect on the carotenoid production as mentioned above, but it was rather smaller than expected. Therefore, we investigated the effects of some organic substances on the production of carotenoids by Mycobacterium smegmatis. In this study, various amino acids were added to the medium at a relatively low concentration (1 mmole/l, as L-form). As seen in Table 5, some amino acids including histidine, glutamic acid, and serine were effective

for the carotenoid production by this bacterium grown on the "hydrocarbon mixture 2". The effect of histidine was more evident in the case of n-hexadecane-grown cells, as shown in Table 6. In both cases, the carotenoid production was approximately doubled by the addition of histidine. These amino acids, which enhanced the carotenoid synthesis, did not stimulate the cell growth.

Table 5. Effect of various amino acids on the production of carotenoids.

Amino acid	Amount added (m mole/l)	Cell yield (g-dry cells/l)	Carotenoid produced (μg/l)	Carotenoid produced (μg/g cells)
None	...	2.78	180	65
L-Glycine	1	2.16	270	125
D, L-Serine	2	1.86	360	194
L-Threonine	1	3.06	170	56
L-Leucine	1	2.31	300	130
L-Isoleucine	1	2.40	240	100
D, L-Cystein	2	2.85	190	67
L-Homocystein	1	2.12	200	94
L-Cystine	1	2.80	170	61
L-Methionine	1	0.65	180	277
L-Tyrosine	1	2.07	320	155
L-Arginine	1	2.54	220	87
L-Histidine	1	2.38	320	135
L-Proline	1	2.01	260	129
D, L-Tryptophan	2	2.41	180	75
L-Phenylalanine	1	1.87	140	75
L-Glutamic acid	1	1.90	350	184
L-Glutamine	1	1.99	190	96
L-Aspartic acid	1	1.79	140	78
L-Asparagine	1	2.26	140	62
L-Lysine	1	2.35	150	64

Cultivation was carried out for 6 days using the "hydrocarbon mixture 2" as a carbon source.

Table 6. Effect of histidine on the carotenoid production.

Amount added (m mole/l)	Cell yield (g-dry cells/l)	Carotenoid produced (μg/l)	(μg/g-cells)
0	3.18	323	102
0.3	3.16	647	205
3.0	3.28	739	225

Cultivation was carried out on a rotary shaker (220 rpm) for 6 days using n-hexadecane as a carbon source.

3.4. Effect of vitamins

Among nine water-soluble vitamins tested, thiamine and biotin showed a good effect on the carotenoid production by Mycobacterium smegmatis (Table 7). Riboflavin, folic acid, cyanocobalamine, Ca-pantothenate, and nicotinic acid were slightly effective, but pyridoxine and p-aminobenzoic acid were not effective. All these vitamins did not affect the bacterial growth on hydrocarbon.

Table 7. Effect of water-soluble vitamins on the carotenoid production.

Vitamin	Amount added (mg/ℓ)	Cell yield (g-dry cells/l)	Carotenoid produced (μg/l)	(μg/g-cells)
None	...	3.04	246	81
Thiamine	1.0	2.89	475	164
Riboflavin	1.0	2.96	292	99
Biotin	0.01	3.15	408	129
Folic acid	0.01	3.07	300	98

Cultivation was carried out for 5 days using "hydrocarbon mixture 2" as a carbon source.

3.5. Effect of surface-detergents

In the hydrocarbon fermentation, it has been generally accepted that emulsification of water-insoluble substrate becomes a limiting factor for bacterial growth. Then the effect of non-ionic detergents on the carotenoid production as well as bacterial growth were investigated. As shown in Table 8, Tween 20, 40, 80, and Span 20 were effective at the concentration of 0.005 and (or)0.0005 %. From these results, we used Tween 80 at the concentration of 0.005 % in the subsequent experiments. These detergents had no influences on the final cell yield as other organic substances.

3.6. Effect of various nitrogen sources

As shown in Table 9, a mixture of urea and $(NH_4)_2SO_4$ was most suitable for the bacterial growth, while $(NH_4)_2CO_3$ or $(NH_4)_2HPO_4$ favored the carotenoid production. A mixture of $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$ or that of $(NH_4)_2CO_3$ and $(NH_4)_2HPO_4$ was also suitable. Thus, the ammonium salts of week acids were found to be appropriate for the carotenoid production. The reason would be ascribed to slight decrease of pH of culture broth during the cultivation in the case of these nitrogen sources.

Table 8. Effect of various non-ionic detergents on the carotenoid production.

Detergent	Amount added (μg/l)	Cell yield (g-dry cells/l)	Carotenoid produced (μg/l)	Carotenoid produced (μg/g cells)
Tween 20	50	3.15	354	112.3
	5	2.94	620	210.9
Tween 40	50	2.75	580	210.9
	5	3.03	514	169.6
Tween 60	50	2.93	309	105.5
	5	3.04	530	174.3
Tween 80	50	2.95	644	218.3
Tween 85	50	2.76	252	91.3
	5	2.93	290	99.0
Span 20	50	2.77	519	187.4
Span 40	50	2.97	324	109.1
Span 60	50	2.66	276	103.8
	5	3.10	270	87.1
Span 80	50	3.17	325	102.5
	5	3.18	332	104.4
None	...	3.06	304	99.3
	...	3.19	352	110.3

Cultivation was carried out on a rotary shaker (220 rpm) at 30°C for 5 days using the "hydrocarbon mixture 3" as a carbon source.

3.7. Effect of aeration

In the hydrocarbon fermentation, aeration is an important factor for the microbial growth as well as emulsification of the substrate. Growth and carotenoid synthesis of Mycobacterium smegmatis were also affected by aeration, as seen in Table 10. The data indicated the existence of the optimal aeration rate

for the carotenoid production. The maximum carotenoid production, 770 μg per liter, was obtained in a 500-ml shaking flask containing 100 ml of the medium shown in Table 1 and 10^{-4} M of L-histidine.

Table 9. Effect of various nitrogen sources on the production of carotenoids.

Nitrogen source	Cell yield (g-cells/l)	Carotenoid produced (μg/l)
$(NH_4)_2SO_4+(NH_2)_2CO$*	3.04	245
$(NH_4)_2SO_4$	0.82	250
$(NH_4)_2CO_3$	2.96	360
$(NH_4)_2HPO_4$	1.34	430
NH_4NO_3	1.31	89
NH_4Cl	1.25	112
$(NH_2)_2CO$	1.91	72
$(NH_4)_2SO_4+(NH_4)_2HPO_4$**	1.71	329
$(NH_4)_2SO_4+(NH_4)_2CO_3$**	1.86	276
$(NH_4)_2CO_3+(NH_4)_2HPO_4$**	2.62	361
Casamino acid	1.22	55

Cultivation was carried out for 5 days using "hydrocarbon mixture 2" as a carbon source.
* $(NH_4)_2SO_4$ 0.15% and $(NH_2)_2CO$ 0.05%, as shown in Table 1.
** These were added at the concentration of 0.1%, respectively. Other nitrogen sources were added at the concentration of 0.2%, respectively.

Table 10. Effect of aeration on the production of carotenoids.

Volume of medium in 500-ml flask	k_La (hr^{-1})	Cell yield (g-cells/l)	Carotenoid produced (μg/l)	(μg/g-cells)
25 ml	237	2.09	379	180
50	149	2.53	645	255
100	66	2.48	770	310
200	36	2.16	400	185

Cultivation was carried out on a rotary shaker (220 rpm) for 6 days at 30°C.
n-Hexadecane was used as a carbon source and 10^{-3} mole per litter of histidine was added.

3.8. Effect of metal ions

Some metal ions are known to be essential in the various enzymic activity and others to be inhibitory. Therefore, we examined the effect of some metal ions in order to obtain larger amount of carotenoids in the hydrocarbon fermentation. As shown in Table 11, omission of ferric ion had serious influences on the bacterial growth, and then on the carotenoid synthesis. Mg^{++} and Ca^{++} were also essential for the growth and carotenogenesis (data were not shown), but Mn^{++} and Co^{++} showed little effect. On the other hand, Zn^{++} and Cu^{++} were inhibitory to the growth and carotenoid production. This tendency was more obvious in the case that distilled or deionized water was substituted for tap water.

4. Discussion

Ciegler et al.[2, 3] reported that β-carotene biogenesis in Blakeslea trispora was markedly enhanced by addition of oily substances, such as kerosene. From this result, they suggested that oily substances would extract β-carotene from growing cells and then would stimulate carotenogenesis of this organism. In hydrocarbon fermentation, it may be supposed that hydrocarbon will make up a complex with carotenoids at the cell membrane and this phenomenon would

Table 11. Effect of metal ions on carotenoid production

Addition or Omission	Water used	Cell yield g cell/l	Carotenoid produced μg/l
None	d*	2.26	1089
	t**	2.66	258
- $FeSO_4.7H_2O$	d	no growth	---
	t	1.79	84
- $MnSO_4.nH_2O$	d	2.21	770
	t	2.66	316
- $CoSO_4.7H_2O$	d	2.72	993
	t	2.88	713
+ $ZnSO_4.7H_2O$	d	2.26	320
	t	0.54	trace
+ $(NH_4)_2Mo_7O_{24}.4H_2O$	d	2.20	576
	t	2.34	180
+ $CuSO_4.5H_2O$	d	2.73	590
	t	1.09	trace

d* : deionized water
t**: tap water
Cultivation was carried out for 6 days using "hydrocarbon mixture 2" as a carbon source.
$ZnSO_4.7H_2O$, $CuSO_4.5H_2O$ and $(NH_4)_2Mo_7O_{24}.4H_2O$ were added at the concentration of 1.0 mg/l. Other medium constituents were shown in Table 1.

favor not only the extraction of carotenoid from cells as supposed by Ciegler *et al.*[2, 3], but also the incorporation of the hydrocarbon into the bacterial cells. Our findings that some bacteria producing carotenoids during hydrocarbon assimilation made up a clump of cells including the substrate, and that carotenoids produced were readily extracted from this clump by *n*-hexane only,[4, 5] may support the view that carotenoids will play some role in hydrocarbon assimilation by certain bacteria.

Haas *et al.*[6, 7] reported that *Mycobacterium lacticola* produced three carotenoids including β-carotene in a medium containing hydrocarbons as a sole carbon source, while *Mycobacterium phlei*, *Mycobacterium leprae*, and *Mycobacterium smegmatis* did not produce carotenoid in hydrocarbon fermentation. On the contrary, our strain of *Mycobacterium smegmatis* produced at least six carotenoids using *n*-alkanes as well as glucose as carbon sources, and these carotenoids did not include a detectable amount of β-carotene.

In this study, the maximum yield of carotenoids by *Mycobacterium smegmatis*, estimated as β-carotene, was about 1 mg per liter, and this figure was very small as compared with the data given by Ciegler *et al.*[2, 3]. But it has been said that xanthophylls are superior to β-carotene as

coloring agents for foods. Therefore, it is interesting to produce a large amount of xanthophylls by fermentation process, especially using hydrocarbon substrates. We could increase the yield of carotenoids by two- to three-fold by addition of histidine and Tween 80. In carotenogenesis, it has been said that Mg^{++} and (or) Mn^{++} are essential[8,9] and Cu^{++} are inhibitory. In the case of Mycobacterium, Mg^{++}, Fe^{+++}, and Ca^{++} was essential for the growth and the carotenoid synthesis. Presumably, Fe^{+++} would participate initial oxidation of hydrocarbons (Oxygenase system) and Mg^{++} would have a role in the condensation of C_5 units. On the contrary, Zn^{++} and Cu^{++} were inhibitory. Elimination of these heavy metal ions from culture medium by deionization enhanced the cell growth and the carotenoid synthesis. The effects of amino acids on the carotenoid production would be partly due to the ability to form complex with these heavy metals as suggested by Sarker et al.[10]

5. Summary

In this chapter, we dealt with the effects of medium constituents, aeration, and addition of some organic compounds on the carotenoid production by Mycobacterium smegmatis. This bacterium could grow on n-alkanes of a medium chain-length

(C_{11-19}), but not on those with shorter chains under the cultural condition used. Among pure n-alkanes, n-hexadecane was most suitable for bacterial growth and the carotenoid production, but "hydrocarbon mixture 2" (rich in n-undecane) was also suitable for carotenoid production. Addition of surface detergent, Tween 80, and amino acids, such as histidine, glutamic acid, and serine stimulated the carotenoid production by Mycobacterium smegmatis. Elimination of heavy metal ions from the medium was effective for carotenoid synthesis. Under the cultural condition tested, the maximal carotenoid production was about 1 mg per liter of medium.

6. References

1). G. Katsui : Bitamin-Gaku no Shimpo, Vol. 4, 115 (Japan Vitamin Society, Kyoto) (1962)

2). A. Ciegler, M. Arnold and R.F. Anderson : Appl. Microbiol., 7, 98 (1959)

3). A. Ciegler, E.N. Nelson and H.H. Hall : Appl. Microbiol., 10, 132 (1962)

4). T. Nagasaki, K. Fujii, A. Tanaka and S. Fukui : Proc. of the 18th Annual Meeting of Japan Vitamin Soc. (Osaka) (May 11, 1966)
Vitamins (Kyoto), 34, 146 (1966)

5). S. Fukui, A. Tanaka, K. Fujii and T. Nagasaki : Proc. of the 40th Annual Meeting of Japan Biochem. Soc. (Osaka) (Nov. 6, 1967)
Seikagaku (Tokyo), 39, 532 (1967)

6). H. F. Haas, M.F. Yanzi and L.D. Bushnell : Transaction Kansas Acad. Sci., 44, 39 (1941)

7). H.F. Haas and L.D. Bushnell : J. Bacteriol., 48, 219 (1944)

8). Y. Kakutani : J. Biochem. (Tokyo), 62, 179 (1967)

9). J.M. Charlton, K.J. Treharne and T.W. Goodwin : Biochem. J., 105, 205 (1967)

10). B. Sarkar and Y. Wigfield : J. Biol. Chem., 242, 5572 (1967)

Chapter 2. Isolation and characterization of several carotenoids produced by *Mycobacterium smegmatis*

1. Introduction

Haas *et al.*[1] reported on the growth and the carotenoid production of *Corynebacterium* and *Mycobacterium* in media containing various hydrocarbons as sole carbon source. They isolated three carotenoids from *Mycobacterium lacticola* and four carotenoids from *Corynebacterium*. In either case the formation of β-carotene was demonstrated. Furthermore, it has been reported that *Nocardia lutea* and *Nocardia corallina* grown on n-hexadecane[2], and *Pseudomonas methanica* grown on methane[3] produced carotenoids or carotenoid-like substances. Ciegler *et al.*[4,5] observed an enhancing effect of fatty substances or hydrocarbons on β-carotene production by *Blakeslea trispora* in carbohydrate media and speculated that these substances served as a storage- and extracting-solvent of β-carotene during the cultivation of this mold. On the other hand, Haas *et al.*[6] reported that *Mycobacterium phlei*, *Mycobacterium leprae*, and *Mycobacterium smegmatis* which could grow on hydrocarbons, did not produce carotenoid when hydrocarbons were used as sole carbon source. But our strain of *Mycobacterium smegmatis* could produce relatively large

amount of carotenoids when cultured on various hydrocarbon substrates, as reported in the preceding chapter[7]. A preliminary experiment[8] has indicated that the carotenoids produced by this bacterium were mainly composed of 4-keto-γ-carotene and its derivatives exhibiting a contrast to the carotenoids produced by Mycobacterium lacticola which included β-carotene.[1] 4-Keto-γ-carotene and its derivatives have been reported by Hertzberg and Jensen[9,10] as minor components of the carotenoids produced by Mycobacterium phlei grown on non-hydrocarbon substrate. In the preceding chapter, we described about the cultural conditions for carotenoid production by Mycobacterium smegmatis. This chapter deals with the structure of these carotenoids.

2. Experimental procedure

2.1. Microorganism

The organism used in this study was Mycobacterium smegmatis IFO 3080.

2.2. Cultivation method

The cells preincubated in 500-ml shaking flasks were transferred into 10-l jar fermentor containing 6 l of a

medium shown in Table 1, and cultured at 30°C with continuous agitation (400 rpm) and aeration (10 l of air per min). After 4 days' cultivation, the cultured filtrate alone was removed from fermentor and 6 l of the fresh medium containing 3.0 % of "hydrocarbon mixture 1"[11] was added aseptically and the second cultivation was carried out under the same cultural condition mentioned above, for 2 days. The third and fourth cultivations were successively carried out for 2 days, respectively, under the same cultural condition except that 4.0 % of the substrate was added instead of 3.0 %. After total 10 days cultivation from the initial inoculation, cells were harvested. By this procedure, we obtained 52 g of cells and 13 mg of carotenoids.

Table 1. Composition of hydrocarbon medium used.

$(NH_4)_2HPO_4$	2.6 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$FeSO_4 \cdot 7H_2O$	0.005g
$MnSO_4 \cdot nH_2O$	0.005g
$CoSO_4$	0.001g
$CaCl_2 \cdot 2H_2O$	0.01 g
KH_2PO_4	2.0 g
$Na_2HPO_4 \cdot 12H_2O$	3.0 g
L-Histidine. HCl	0.192g
Hydrocarbon	20 ml
Tap water	1000 ml
pH	7.0

2.3. Extraction of carotenoids and removal of residual substrate

The cells harvested were washed with water and a small amount of acetone, successively, and then were extracted with about three volumes of petroleum ether for 10 hrs. This and following procedures were carried out in the dark and in N_2 atomosphare. In this petroleum layer, about eighty per cent of the total carotenoids produced were extracted. After centrifugation, the resulted petroleum ether layer was passed through a column packed with silica-gel (100 mesh) (purchased from Mallinckrodt Chemical Works, St. Louis), and pigments adsorbed were extruded from this column and eluted with acetone. With this treatment residual substrate and most of waxy substances produced were removed. On the other hand, cells sedimented by the centrifugation were further extracted twice with about three volumes of acetone-petroleum ether (1 : 1) for 15 hrs. To this extracts, a small volume of water was added without shaking in order to separate the petroleum ether layer from acetone-water layer. The petroleum ether layer thus obtained was combined with the above-mentioned pigments purified partially by silica-gel chromatography, evaporated to dryness <u>in vacuo</u> and then dissolved in acetone. The resulted acetone solution of carotenoids was allowed to stand at -20°C for 5 hrs to remove waxy substances which precipitated as white crystals. These treatments to remove

the residual hydrocarbon substrate and waxy substances produced were necessary for the further purification of carotenoids.

2.4. Saponification

The carotenoid mixture obtained above was dissolved in benzene and saponified using two volumes of 10 % KOH in methanol for 30 min at 40°C with continuous stirring in N_2 atomosphare. The unsaponifiable fraction containing carotenoid pigments was extracted with petroleum ether, and washed five times with water, and evaporated to dryness *in vacuo*, then stored at -20°C in N_2 atomosphare.

2.5. Fractionation of carotenoids

The carotenoid mixture was adsorbed on the top of a silica-gel column and was fractionated into two zones when developed with petroleum ether. The yellow zone migrated slowly and the red zone was retained on the top of the column. Each of these two pigmented zones was extruded from the column and eluted with acetone.

The yellow pigment (Fr. 0) was further separated by aluminium oxide paper chromatography as follows. That is, using petroleum ether containing a trace amount of benzene as developing solvent, a fluorescent substance having the R_f

value of 0.90-0.95 and yellow substance staying at the origin were detected. This fluorescent substance was named as Fr. 0-a. The yellow pigments were further separated by aluminium oxide paper, using benzene-petroleum ether(15 : 85) as solvent, into two yellow zones which had the R_f values of 0.50 (Fr. 0-b) and 0.10 (Fr. 0-c), respectively.

The red zone obtained in the first silica-gel column chromatography (Fr. I) was rechromatographed on silica-gel with acetone-petroleum ether (5 : 95) as solvent. Two zones were obtained, that is, an orange pigment eluted with this solvent from the column (Fr. I-a) and a red pigment retained on the top of the column (Fr. I-b). This pigment was extracted with acetone from the adsorbent.

Fr. I-a was further chromatographed on a column of silica-gel-$Ca(OH)_2$ (1 : 2) with acetone-petroleum ether (2 : 98) and two pure carotenoids were obtained (Fr. 1 and 2).

On the other hand, when Fr. I-b was chromatographed on a column of silica-gel-$Ca(OH)_2$ (1 : 2) with acetone-petroleum ether (7 : 93), one pure carotenoid (Fr. 3) and a carotenoid mixture were obtained. This mixture was separated on a column of silica-gel-$Ca(OH)_2$ (1 : 1) using an increasing concentration of acetone in petroleum ether as developer into Fr. 4, Fr. 5, and Fr. 6, and with benzene-acetone (99 : 1) into Fr. 7 and

Fr. 8.

The isolated carotenoids were further purified by aluminium oxide paper chromatography. These separation procedures were summarized in Table 2.

Table 2. Chromatographic separation of the carotenoids produced by Mycobacterium smegmatis.

Fraction No.	Column chromatography				Aluminium oxide paper chromatography
	Silica-gel	Silica-gel	Silica-gel-$Ca(OH_2)$ (1:2)	Silica-gel-$Ca(OH)_2$ (1:1)	
0-a	Migrated with PE. Extruded and eluted with Ac				Developed with B-PE
0-b					
0-c					
1		Eluted with Ac-PE(5:95)	Eluted with Ac-PE(2:98)		
2					
3	Not migrated with PE. Extruded and eluted with Ac		Eluted with Ac-PE(7:93)		
4					
5		Extruded and eluted with Ac	Extruded and eluted with Ac	Eluted with Ac-PE	
6					
7					
8				Eluted with Ac-B(1:99)	

Ac: acetone, B: benzene, PE: petroleum ether.

All fractions were further purified by aluminum oxide paper chromatography.

2.6. Characterization of carotenoids

2.6.1. Absorption spectrum

Shimadzu MPS-50 Type Multi-purpose Spectrophotometer was used to obtain visible and ultraviolet absorption spectra.

2.6.2. Aluminium oxide paper chromatography

Aluminium oxide paper[12] (Schleicher and Schüll, No.288) was used in order to separate, identify, and judge the purity of carotenoids. Several combinations of acetone, benzene and petroleum ether were used as developing solvents.

2.6.3. Reduction of carbonyl group

This treatment was performed in order to study the presence of carbonyl group(s) in the carotenoids obtained. It is well known that carotenoids containing carbonyl groups are converted to the corresponding hydroxy-carotenoids when reduced with small amounts of $NaBH_4$ in their 95 % ethanol solution. The formation of hydroxy-carotenoids results in a change in the relative polarity and, in the case of allylic carbonyl groups, results in increased fine structures and a hypsochromic shift in the visible absorption spectrum.

2.6.4. Acetylation of hydroxy-carotenoids

This procedure are used to distinguish primary and secondary hydroxy groups from tertiary hydroxy groups[13]. The carotenoids dissolved in dry pyridine are treated with acetic anhydride and reacted at room temperature overnight, then extracted with diethyl ether. Only the primary and secondary hydroxy groups are acetylated under these conditions.

2.6.5. Acid chloroform test

Carotenoids containing allylic hydroxy groups are

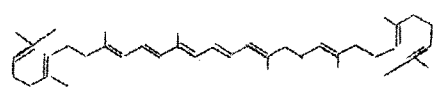
dehydrated when their chloroform solutions are treated with small amounts of dry HCl-containing chloroform[14]. This treatment yields a carotenoid having one more conjugated double bond and bathochromic shift in the visible absorption spectrum and also a decreased relative polarity. Allylic alkoxy groups give the same results.

3. Results and discussion

The carotenoids extracted from the cells of Mycobacterium smegmatis were separated into eleven components by silica-gel and silica-gel-$Ca(OH)_2$ column chromatography and purified by aluminium oxide paper chromatography. These separation procedures were summarized in Table 2. The carotenoids thus separated were classified into four categories, that is, (1) precursors of carotene biosynthesis, including phytofluene, ζ-carotene, and neurosporene, (2) 4-keto-γ-carotene and its derivatives, (3) derivatives of 3', 4'-dehydro-4-keto-γ-carotene, and (4) pigments having unidentified chromophores. Some of these carotenoids were identified by their visible absorption spectra as well as their behavior on aluminium oxide paper chromatography, before and after the chemical treatments mentioned above.

3.1. Fraction O-a

This fluorescent substance had the absorption maxima at 330, 347, and 367 mμ in petroleum ether, which were in good agreement with those of phytofluene reported by Treharne et al.[15] (Table 3). The absorption spectrum of Fr. O-a was shown in Fig. 1.



Phytofluene

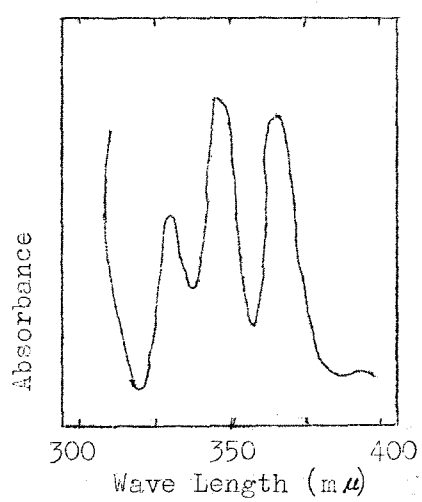
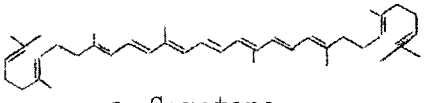


Fig. 1. Absorption spectrum of Fr.O-a in petroleum ether.

3.2. Fraction O-b

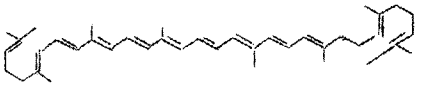
This fraction was not obtained in the pure state, but the absorption maxima at 358, 378, 400, and 425 mμ in petroleum ether were fairly agreed with those of ζ-carotene[16,17] as shown in Table 3.



ζ-Carotene

3.3. Fraction O-c

This fraction showed an absorption maximum at 445 mμ in petroleum ether and was identified as neurosporene[15].



Neurosporene

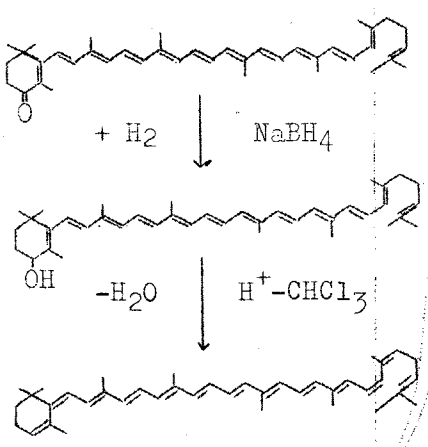
The absorption maxima of these three carotenoids were summarized in Table 3 together with those of the authentic ones.

Table 3. Comparison of absorption maxima of Fr. O-a, O-b and O-c with those of authentic ones.

Carotenoid	Absorption maxima in petroleum ether (mμ)
Fr. O-a	330, 347, 367
Fr. O-b	358, 378, 400, 425
Fr. O-c	445
Phytofluene[15]	330, 347, 367
ζ-Carotene[16,17]	362, 379, 400, 425
Neurosporene[15]	445

3.4. Fraction 1 and 2

These two carotenoids showed very similar behaviors on the column chromatography and in chemical treatments. Fr. 2 had a broad spectrum (λ_{max} 467 mμ in petroleum ether) which changed into a fine structure (λ_{max} 435, 460, and 489 mμ in petroleum ether) and showed about 7 mμ of hypsochromic shift (Fig. 2) when the sample was reduced with $NaBH_4$. This change indicated that Fr. 2 would have a conjugated carbonyl group on its ionone ring. Furthermore, reduced Fr. 2 showed about 23 mμ of bathochromic shift when treated with acid chloroform, and this suggested the formation of a dehydrated product and the existence of an allylic hydroxy group as shown below. On the other hand,



the absorption spectrum of reduced Fr. 2 was the same as that of γ-carotene[17], and that of dehydrated Fr. 2 was very similar to that of retro-dehydro-γ-carotene[10] (Fig. 2 and Table 4). Thus, it was concluded that Fr. 2 had the same chromophore as that of 4-keto-γ-carotene. The presence of other substituent was not precluded, but the IR-spectrum of this fraction showed the absence of any substituent (Fig. 3). The IR-spectrum also indicated that Fr. 2 would be all-trans isomer; that is, the absence of the absorption at 780 cm^{-1} and the non-branching of the absorption at 960 cm^{-1} (Fig. 3) would deny the cis-configulation. From these results, we concluded that Fr. 2 was 4-keto-γ-carotene.

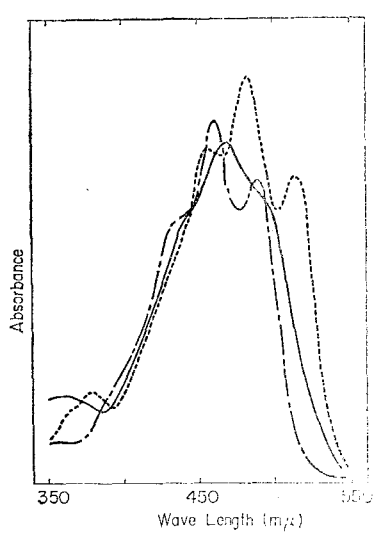
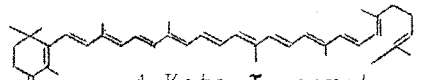


Fig.2. Absorption spectra of Fr.2 and its derivatives in petroleum ether.
(——) : Fr. 2
(— —) : reduced Fr. 2
(---) : dehydrated Fr. 2

Fr. 1 showed the same behavior as that of Fr. 2, but its absorption spectrum differed somewhat. Fr. 1 had the absorption maximum at 460 mμ in petroleum ether, and the reduced one 350, 435, 455.5, and 484 mμ in petroleum ether (Table 4). The absorption at 350 mμ suggested that this carotenoid would have a cis-configuration. The cis isomer of γ-carotene is known to show the same absorption spectrum as that of reduced Fr. 1. From these results, we confirmed the identification of Fr. 2 as all-trans 4-keto-γ-carotene and Fr. 1 as cis isomer of 4-keto-γ-carotene. 4-Keto-γ-carotene might have been converted into its cis isomer during the cultivation of bacterium.



4-Keto-γ-carotene

Table 4. Properties of Fr. 1, 2 and their derivatives.

Carotenoid	Absorption maxima (mμ)			R_f value*
	in petroleum ether	in acetone	in chloroform	
Fr.1	460	463.5		
Reduced Fr.1	350,435,455,484	350,437,459,487		
Fr.2	467	470	482	0.7
Reduced Fr.2	435,460,489	440,465,495	450,473,501	0.5
Dehydrated Fr.2	379,456,483,514	382,465,489,520	390,473,499,532	1.0
4-Keto-γ-carotene		471,490		
γ-Carotene		440,465,495		
γ-Carotene(cis)	455,486	351,437,458,487		
Retro-dehydro-γ-carotene		380,462,487,518		

* Aluminium oxide paper chromatography (solvent: benzene)

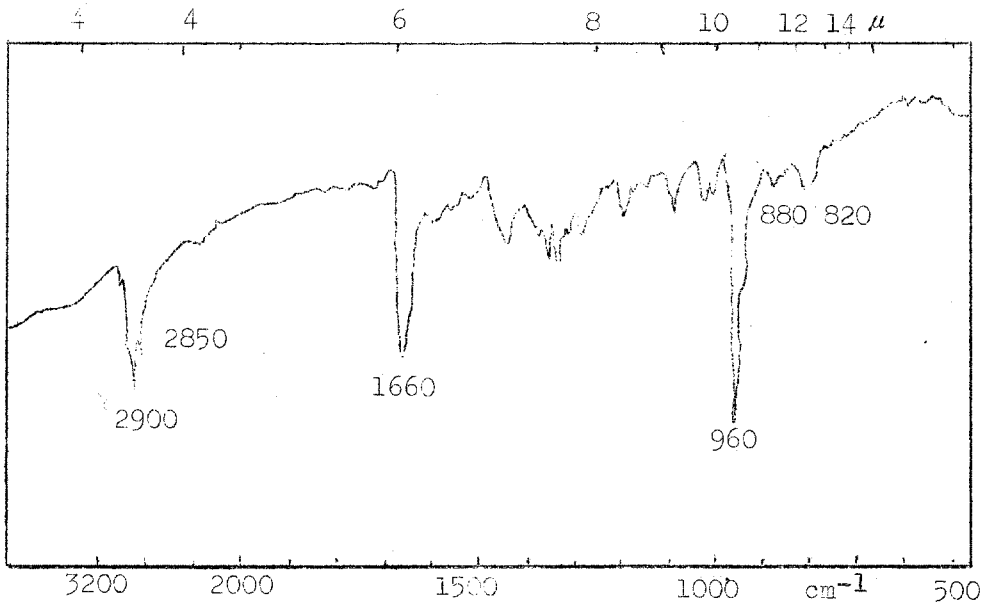
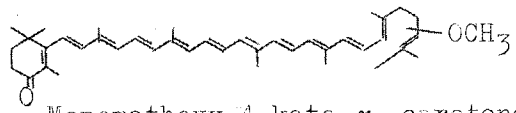


Fig. 3. IR-spectrum of Fr. 2 (KBr tablet).

3.5. Fraction 3

The absorption spectrum of this fraction was very similar to that of Fr. 2. Then, this carotenoid would have the same chromophore as that of 4-keto-γ-carotene. The polarity of this carotenoid was larger than that of 4-keto-γ-carotene, but was the same as that of 4-hydroxy-γ-carotene. Fr. 3 had a conjugated carbonyl group since the broad absorption spectrum of this fraction changed into the fine structure when treated with $NaBH_4$ (Fig.4 and Table 5). These results suggested that this carotenoid would have one more substituent such as methoxy or epoxy group on the structure of 4-keto-γ-carotene. But this fraction showed the negative result on the test for epoxide detection. Although we could not determine the position of methoxy group, we gave the structure of monomethoxy-4-keto-γ-carotene for this fraction.



Monomethoxy-4-keto-γ-carotene

3.6. Fraction 6

The absorption spectrum of this fraction was similar to that of 4-keto-γ-carotene (Table 5). From the polarity data, this carotenoid seemed to have one conjugated carbonyl group and one hydroxy group. Although we could not identify this

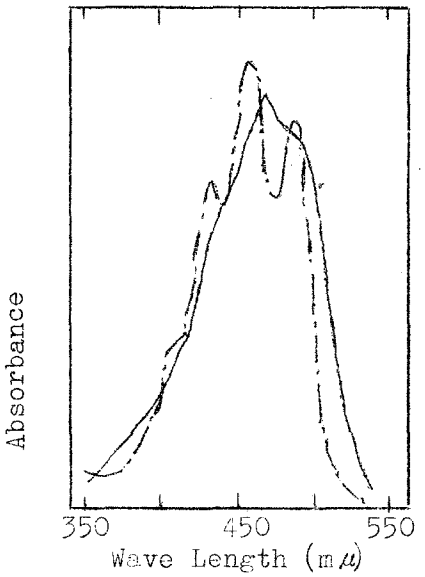
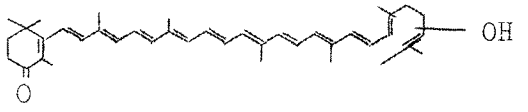


Fig.4. Absorption spectra of Fr.3 and reduced Fr. 3 in petroleum ether.
(——) : Fr. 3
(– – –) : reduced Fr. 3

carotenoid exactly, the compound was likely to be monohydroxy-4-keto-γ-carotene.

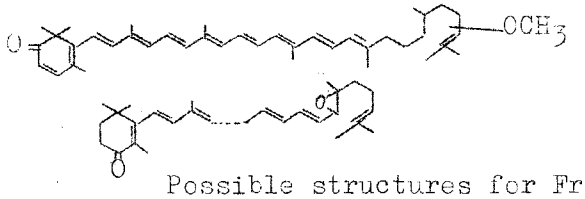


Monohydroxy-4-keto-γ-carotene

3.7. Fraction 4

The absorption maximum at 450 mμ in petroleum ether of

this fraction indicated that the number of the conjugated double bonds of the compound was one less than that of 4-keto-γ-carotene. The fine structure of reduced Fr. 4 also showed the presence of one conjugated carbonyl group (Table 5). From the behavior on column chromatography, this carotenoid would have one more substituent, such as alkoxy or epoxy group. This fraction showed the positive epoxide test when treated with acid ether, but further identification was not carried out. The possible structure of Fr. 4 is supposed to be one of the following two structures.



Possible structures for Fr.4

Table 5. Absorption maxima of Fr. 3,4, and 6, and R_f value of Fr. 3.

Fraction	Absorption maxima (mμ)		R_f value* (solvent)
	in petroleum ether	in acetone	
Fr. 3	466	470	0.5(with PE-B**)
Reduced Fr.3	433,458,488	437,462,492	0.8(with Ac-B***)
Fr. 4	450,465		
Reduced Fr.4	42),442,471		
Fr. 6	462.5	467	

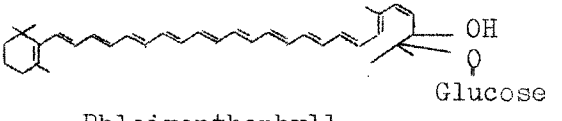
* Aluminium oxide paper chromatography

** Petroleum ether-benzene (15:85)

*** Acetone-benzene (3:35)

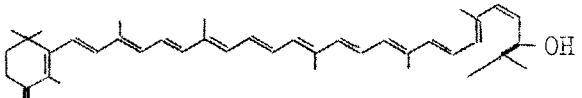
3.8. Fraction 7 and 8

The absorption maximum of Fr. 8 in chloroform was at 494 mμ , and this indicated that this carotenoid would have one more elongated chromophore compared to that of 4-keto-γ -carotene. The change of the broad absorption spectrum of Fr.8 into the fine structure also showed the presence of one conjugated carbonyl group on its ionone ring (Fig. 5 and Table 6). On acetylation, this fraction gave only one acetylated derivative, that is this carotenoid had only one secondary hydroxy group. This hydroxy group was removed when treated with acid chloroform, and so was confirmed as allylic hydroxy group. These results were ascertained by the aluminium oxide paper chromatography with acetone-benzene (1 : 9) as solvent. Furthermore, phleixanthophyll, reported by Jensen[10] as a component of mycobacterial carotenoids, showed the very similar absorption spectrum as that of reduced Fr. 8. From these results, we gave the structure of 1', 2'-dihydro-2'-hydroxy-3', 4'-dehydro-4-keto- γ-carotene for Fr. 8.



Phleixanthophyll

Fr. 7 showed very similar behavior to Fr. 8 in the absorption spectrum and in the chemical treatment such as reduction, acid chloroform treatment and acetylation. The absorption maximum of Fr. 7 at about 350 mμ indicated that this fraction would be a cis isomer of Fr. 8. Fr. 8 might have been converted into Fr. 7 during the cultivation as the case of Fr. 1 and 2.



1',2'-Dihydro-2'-hydroxy-3',4'-dehydro-4-keto-γ-carotene

Thus we could characterize several carotenoids produced by Mycobacterium smegmatis in hydrocarbon fermentation, that is, Fr. 0-a as phytofluene, Fr. 0-b as ζ-carotene, Fr. 0-c as neurosporene, Fr. 1 as 4-keto-γ-carotene (cis isomer), Fr. 2 as 4-keto-γ-carotene, Fr. 3 as monomethoxy-4-keto-γ-carotene, Fr. 6 as monohydroxy-4-keto-γ-carotene, Fr. 7 as 1', 2'-dihydro-2'-hydroxy-3', 4'-dehydro-4-keto-γ-carotene (cis isomer), and Fr. 8 as 1', 2'-dihydro-2'-hydroxy-3', 4'-dehydro-4-keto-γ-carotene.

The cis isomer (Fr. 1 and Fr. 7) would be the secondary products of this bacterium which might be caused by the

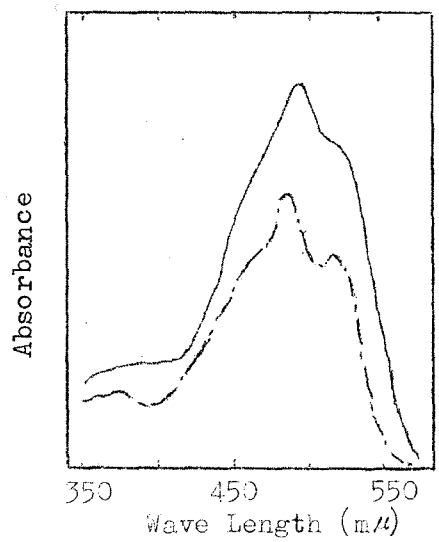


Fig. 5. Absorption spectra of Fr.8 and reduced Fr. 8 in chloroform.
(——) : Fr. 8
(— —) : reduced Fr. 8

Table 6. Absorption maxima of Fr. 7 and 8 and their dervatives.

Fraction	Absorption maxima (mμ)	
	in acetone	in chloroform
Fr. 7	481	(350),493
Reduced Fr. 7	477,505	
Fr. 8	479	494
Reduced Fr. 8		(463),487,520

aeration and agitation during the cultivation. In addition, about eighty per cent of the carotenoids produced were extracted into the hydrocarbon substrate layer during cultivation as mentioned above. The carotenoids extracted into the hydrocarbon layer would be more susceptible to the effect of oxygen or light than the carotenoids existed in the bacterial cells, and might be converted to their cis isomers.

From the results mentioned above, the author proposed the metabolic pathway shown in Fig. 6 for the Mycobacterial carotenoids produced in a hydrocarbon medium.

It is very interesting that Mycobacterium smegmatis grown on hydrocarbons produced 4-keto-γ-carotene and its derivatives as the main components contrary to Mycobacterium lacticola grown on hydrocarbons which produced β-carotene[1] and to Mycobacterium phlei grown on nonhydrocarbon substrate which produced 4-keto-γ-carotene and its derivatives as only minor components[9,10].

4. Summary

This chapter dealt with the isolation and characterization of several carotenoids produced by Mycobacterium smegmatis in hydrocarbon fermentation. These carotenoids were classified into four categories; (1) intermediates in the carotenoid

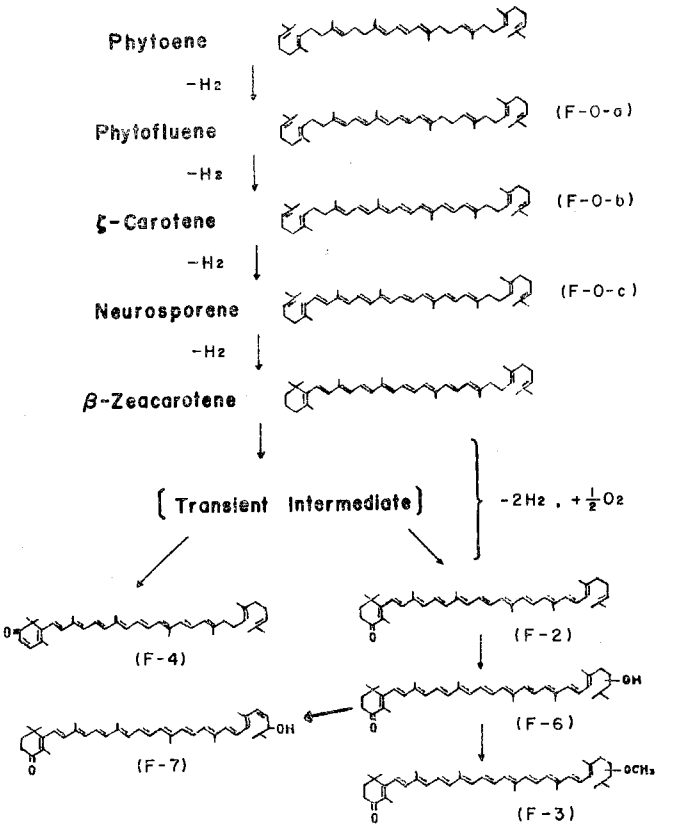


Fig. 6. Proposed carotenogenesis of Mycobacterium smegmatis on hydrocarbon medium.

biosynthetic route, such as phytofluene, ζ-carotene, and neurosporene, (2) 4-keto-γ-carotene and its derivatives (containing eleven conjugated carbon-carbon double bonds and one conjugated carbonyl group), (3) derivatives of 3', 4'-dehydro-4-keto-γ-carotene (containing twelve conjugated carbon-carbon double bonds and one conjugated carbonyl group), and (4) pigments having unknown chromophore. When carotenoids were extracted at the stationary phase, the major part consisted of 4-keto-γ-carotene and its derivatives, presumably monohydroxy- and monomethoxy-4-keto-γ-carotene. Thus we could isolate the precursors of 4-keto-γ-carotene, 4-keto-γ-carotene itself and its derivatives, but could not detect γ-carotene or 4-hydroxy-γ-carotene, the presumable precursors of 4-keto-γ-carotene biosynthesis.

5. References

1). H.F. Haas, M.F. Yanzi and L.D. Bushnell: Transaction Kansas Acad. Sci., 44, 39 (1941)

2). T. Nagasaki, K. Fujii, A. Tanaka and S.Fukui: Proc. of the 18th Annual Meeting of Japan Vitamin Soc. (Osaka) (May 11, 1966)
Vitamins (Kyoto), 34, 146 (1966)

3). M. Dworkin and J.W. Foster: J. Bacteriol., 72, 646 (1956)

4). A. Ciegler, M. Arnold and R.F. Anderson: Appl. Microbiol., 7, 98 (1959)

5). A. Ciegler, G.E.N. Nelson and H.H. Hall: Appl. Microbiol., 10, 132 (1962)

6). H.F. Haas and L.D. Bushnell: J. Bacteriol., 48, 219 (1944)

7). A. Tanaka, T. Nagasaki, M. Inagawa and S. Fukui: J. Ferment. Technol., 46, 468 (1968)

8). T. Nagasaki, A. Tanaka and S. Fukui: Proc. of the 20th Annual Meeting of Japan Chem. Soc. (Tokyo) (April 3, 1967)

9). S. Hertzberg and S.L. Jensen: Acta Chem. Scand., 20, 1187 (1966)

10). S. Hertzberg and S.L. Jensen: Acta Chem. Scand., 21, 15 (1967)

11). A. Tanaka and S. Fukui: J. Ferment. Technol., 46, 214 (1968)

12). A. Jensen: Acta Chem. Scand., 13, 138 (1959)

13). B.P. Schimmer and N.I. Krinsky: Biochemistry, 5, 1814 (1966)

14). P. Karrer and E. Leumann: Helv. Chim. Acta, 34, 445 (1951)

15). K.J. Treharne, E.I. Mercer and T.W. Goodwin: Phytochem., 5, 581 (1966)

16). G. Katsui: Bitamin-Gaku no Shimpo, Vol. 4, 115 (Japan Vitamin Society, Kyoto) (1962)

17). R.J.H. Williams, G. Britton, J.M. Charlton and T.W. Goodwin: Biochem. J., 104, 767 (1967)

Chapter 3. Electromicroscopical study on hydrocarbon-grown and glucose-grown Mycobacterial cells

1. Introduction

The carotenoid pigments of hydrocarbon-grown cells of Mycobacterium smegmatis were more readily extracted with organic solvents than those of glucose-grown cells, as mentioned in the preceding chapter. This phenomenon suggested that the carotenoid pigment would have some role in the incorporation of hydrocarbon substrate into bacterial cells, and that the morphology, especially the construction of cell membrane, of hydrocarbon-grown cells would differ from that of glucose-grown cells, since it is well known that the carotenoid pigments are distributed over the cell envelope rather than in the cytoplasm.[1,2] Furthermore, Ciegler et al.[3,4] demonstrated the extraction of β-carotene by fatty substances from a glucose-grown mold cells. Thus, it is interesting to study on the interaction between intracellular carotenoids and extracellular hydrocarbons in regard to the production of carotenoids on a hydrocarbon medium and to the incorporation of hydrocarbon substrates into bacterial cells. This chapter deals with the electromicroscopical study using hydrocarbon-grown and glucose-grown cells of

Mycobacterium smegmatis.

2. Experimental procedure

2.1. Microorganism

The organism used in this study was Mycobacterium smegmatis IFO 3080.

2.2. Cultivation method and estimation of carotenoid content

Cultivation of the bacterium and estimation of carotenoid content were same as those shown in Chapter 1 of this part.

2.3. Preparation of specimens for electron microscopy

Cells harvested from the cultured broth were sonicated at 10 kc for 15 sec with a detergent in order to remove a residual hydrocarbon substrate, and then were washed twice with saline to remove a detergent. The washed cells were fixed for 15 hrs with 5 % glutaraldehyde in 50 mM phosphate buffer, pH 7.4,[5] washed thoroughly with water, fixed for 2 hrs with 1 % osmium tetraoxide in 50 mM phosphate buffer, pH 7.4,[6] dehydrated successively with 70, 80, 90, 99 and 100 % ethyl alcohol

(tree times) for 15 min, respectively, treated three times with propylene oxide for each 15 min, propylene oxide-Epon 812 (1 : 1) for 30 min, and twice with propylene oxide-Epon 812 (1 : 2) for each 1 hr, and then embedded in Epon 812 according to Luft's schedule.[7,8] In some cases, ultra-thin-section thus obtained were stained with uranyl acetate.

3. Results and discussion

3.1. Extractability of carotenoids produced by Mycobacterium smegmatis

We observed that some bacteria producing carotenoids in their cells made up a clump of cells occluding hydrocarbons when grown on hydrocarbons as a sole carbon source. The carotenoid pigments thus formed were easily extracted with a non-polar solvent, such as n-hexane, from the clump (Fig. 1A and 1C), although amount of total carotenoids produced by hydrocarbon-grown and glucosegrown cells were nearly the same when being extracted with n-hexane-methyl alcohol after both cells were sonicated at 10 kc for 10 min (Fig. 1B). This suggested that the carotenoid pigments locating near the cell membrane of hydrocarbon-grown cells would mediate the incorporation of hydrocarbons into bacterial cells and that

hydrocarbons would act as solvents for carotenoid extraction from bacterial cells, vice versa. The latter situation was demonstrated by Ciegler et al.[3,4] when a mold, Blakeslea trispora, was cultured on glucose as a sole carbon source with addition of fatty substances. Although the role of carotenoids in non-photosynthitic bacteria has not been made clear yet, they may participate in offering the lipophylic field to the metabolism of fatty substances.

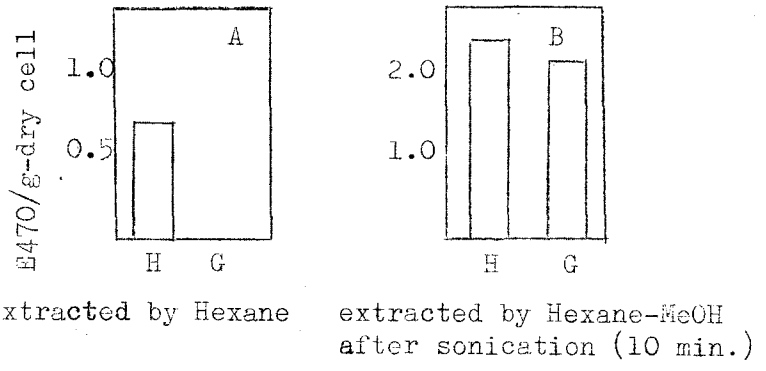


Fig. 1. Extraction of carotenoid pigment produced by Mycobacterium smegmatis under various conditions.

3.2. Electromicroscopical study on hydrocarbon-grown and glucose-grown Mycobacterial cells

In order to obtain some informations about the interaction between the hydrocarbon substrate and cellular carotenoids, the electromicroscopical study was carried out using hydrocarbon-grown and glucose-grown cells of Mycobacterium smegmatis[9]. As shown in Fig. 2, 3 and 4, the size of cells and the thickness of cell wall were nearly the same in both cells. However, some differences were observed between hydrocarbon-grown and glucose-grown cells; that is, the former was round-shaped and its cell membrane was somewhat obscure compared with the latter. Although we could not demonstrate the participation of carotenoids in hydrocarbon uptake of bacterial cells, slime-like substance was observed at the contact face of two hydrocarbon grown cells, as shown in Fig. 4. This substance was not seen on glucose-grown cells (Fig. 5) and may be responsible for the lipophylic nature of hydrocarbon-grown cells. In some cases, amorphous cell wall was observed on hydrocarbon-grown cells and these cells were rather coagulated compared with glucose-grown cells which existed separately. The nucleus, ribosome and mesosome were observed distinctly in glucose-grown cells, but not clearly in hydrocarbon-grown cells. Poly-β -hydroxybutylate-like

substance was seen only in the electromicrograph of hydrocarbon-grown cells as the low density area. Poly-β-hydroxybutylate has been known as the storage substance of carbon and was demonstrated its occurrence in hydrocarbon-grown Nocardia cells.[10,11] Deposits were also observed in hydrocarbon-grown cells and on their cell membrane. These deposits were considered to be hydrocarbon substrate incorporated into cells and attached to cell membrane.

Thus, we could observe the several interesting characteristics of hydrocarbon-grown Mycobacterial cells, although the participation of carotenoids in hydrocarbon uptake could not be demonstrated at al.

4. References

1). M.M. Mathews and W.R. Sistrom: J. Bacteriol., 78, 778 (1959)

2). W.L. Stephens and M.P. Starr: J. Bacteriol., 86, 1070 (1963)

3). A. Ciegler, M. Arnold and R.F. Anderson: Appl. Microbiol., 7, 98 (1959)

4). A. Ciegler, G.E.N. Nelson and H.H. Hall: Appl. Microbiol., 10, 132 (1962)

5). D.D. Sabatini, K. Bensch and R.J. Barrnett: J. Cell

Biol., 17, 19 (1963)

6). G. Millonig: J. Appl. Phys., 32, 1637 (1961)

7). J.H. Luft: J. Biophys. Biochem. Cytol., 2, 799 (1956)

8). J.H. Luft: J. Biophys. Biochem. Cytol., 9, 409 (1961)

9). S. Fukui, A. Tanaka, K. Fujii and T. Nagasaki: Proc. of the 40th Annual Meeting of Japan Biochem. Soc. (Osaka) (Nov. 6, 1967)

10). J. B. Davis: Appl. Microbiol., 12, 301 (1964)

11). W. C. Trentini and R. P. Starr: J. Bacteriol., 93, 1699 (1967)

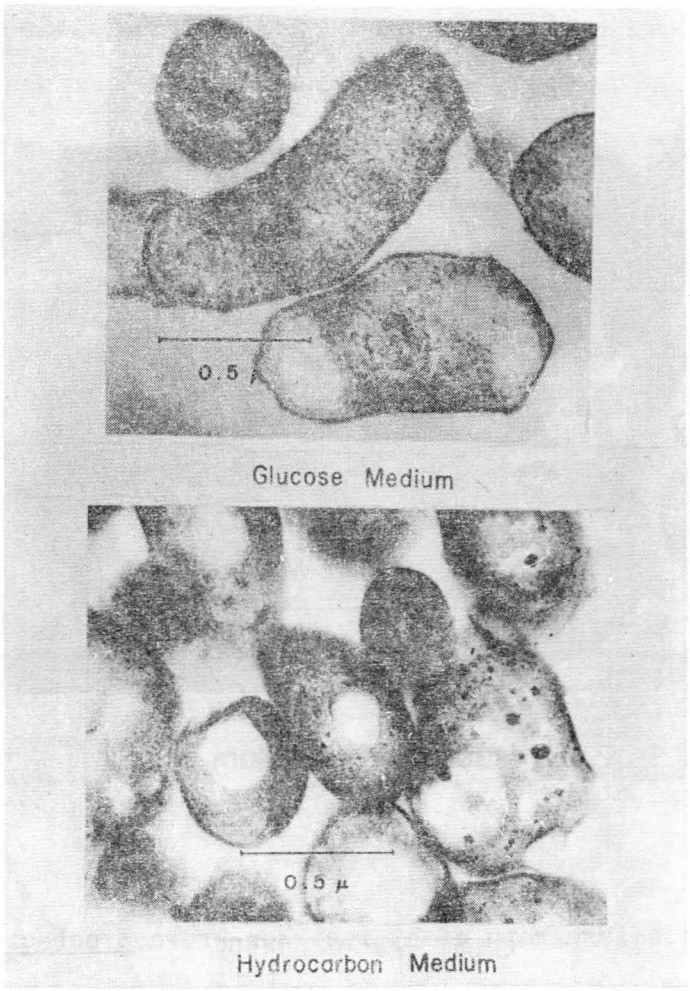


Fig. 2. Electromicrograph of Mycobacterium smegmatis.
Glucose-grown cells (upper) and hydrocarbon-grown cells (lower).
Stained with uranyl acetate.

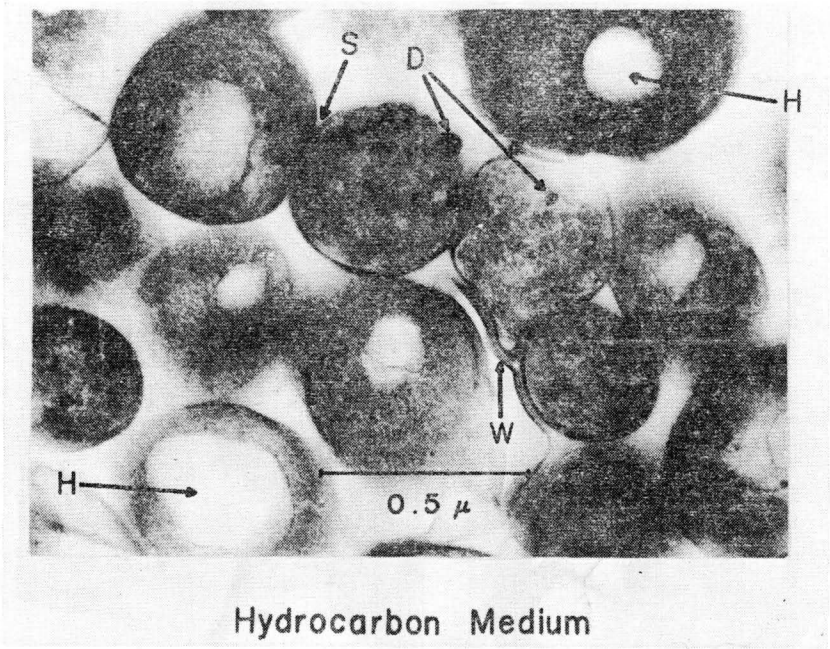


Fig. 3. Electromicrograph of hydrocarbon-grown Mycobacterial cells (unstained).

D : deposit, H : poly-β-hydroxybutylate-like substance, S : slime-like substance, W : cell wall.

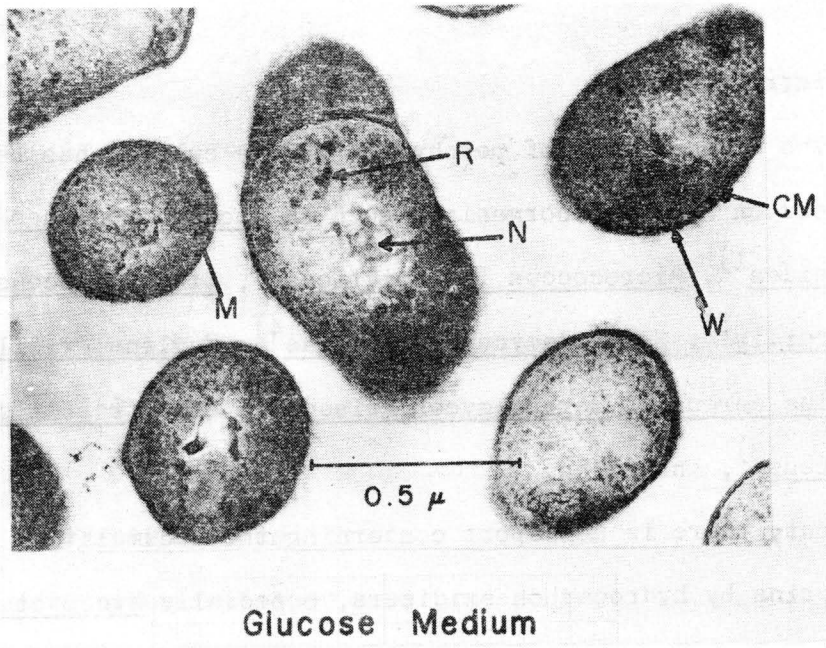


Fig. 4. Electromicrograph of glucose-grown Mycobacterial cells (stained with uranyl acetate).
N : nucleus, M : mesosome, R : ribosome,
CM : cell membrane, W : cell wall.

Part IV. Accumulation of porphyrin in culture filtrate of *Mycobacterium smegmatis* during hydrocarbon assimilation

1. Introduction

The accumulation of porphyrins in the culture has been reported on many microorganisms such as *Rhodopseudomonas spheroides*[1], *Micrococcus lysodeikticus*[2], *Staphylococcus epidermidis*[3], *Saccharomyces cerevisiae*[4], *Euglena gracilis*[5], *Bacillus cereus*[6], *Streptomyces griseus*[7], and *Streptomyces olivaceus*[8], and so on.

But, there is no report concerning the accumulation of porphyrins by hydrocarbon oxidizers, especially *Mycobacterium* species. We found out that *Mycobacterium smegmatis* and *Corynebacterium simplex*, which produced vitamin B_{12} in hydrocarbon fermentation[9], accumulated a large amount of porphyrins in the culture filtrate during hydrocarbon assimilation. Since the biosynthetic route of porphyrins is closely related to that of vitamin B_{12}, then it will be interesting to investigate the type of porphyrins accumulated during hydrocarbon utilization and the relation between the porphyrin accumulation and the vitamin B_{12} production of these bacteria.

In this part, the author will deal with the identification of porphyrin produced by Mycobacterium smegmatis in hydrocarbon fermentation and the cultural conditions affecting its accumulation.

2. Experimental procedure

2.1. Microorganism

The organism used in this study was Mycobacterium smegmatis IFO 3080.

2.2. Cultivation method

Cultivation method of this bacterium was same as that mentioned in Part III. The bacterium was cultured using a 10-l glass fermentor under continuous agitation by vibro-mixer, a 500-ml flask with shaking on a rotary shaker (220 rpm), or a 10-l jar fermentor under continuous agitation (400 rpm) and with adequate aeration, for 6 to 7 days at 30°C[10].

2.3. Hydrocarbon

Hydrocarbons used in this study for the growth substrate of the bacterium were the "hydrocarbon mixture 1" (mixture of n-C_{11-14}) and the "mixture 2" (mixture of n-C_{10-13})[11].

2.4. Extraction of porphyrin

Harvested cells and the culture filtrate were extracted with acetic acid-ethylacetate mixture (1 : 3) several times, respectively, and the combined extracts were washed with deionized water until no acetic acid remained, and then evaporated to dryness *in vacuo* after being dried on sodium sulfate.

2.5. Esterification of porphyrin

The crude porphyrin obtained was esterified with H_2SO_4-methanol (0.5 : 9.5) for 24 hrs at 5-10°C and the resulting methyl ester was extracted with chloroform from ice-chilled reaction mixture, and the extract was washed with deionized water to remove remaining H_2SO_4, and was evaporated to dyness *in vacuo* after being dried on sodium sulfate, according to the method reported by Falk[12].

This porphyrin methyl ester was purified by alumina column chromatography using chloroform-petroleum ether (1 : 3) and chloroform-methanol (100 : 0.5) as developers. Porphyrin methyl ester did not migrate from the top of the column when being developed with chloroform-petroleum ether, but migrated in one red zone in the case of the extract from the culture filtrate and in two red zones in the case of that from cells, when being developed with chloroform-methanol. These red

zones were further purified on the alumina column. The eluates were evaporated to dryness in vacuo and crystallized from chloroform-methanol.

All the solvents used were freshly distilled before use, and alumina was purchased from F. Merck A. G., Darmstadt, Germany.

2.6. Measurement of visible absorption-, infrared- and nuclear magnetic resonance spectra

Visible absorption spectra were measured with Shimadzu MPS-50 Type Multi-purpose Spectrophotometer, and infrared spectra were measured with Hitachi Grating Infrared Spectrophotometer DS-402 G. NMR spectra of the porphyrin methyl ester dissolved in $CDCl_3$ were obtained using Varian NMR Spectrophotometer Model A 60.

2.7. Paper chromatography

Toyo Roshi No. 50 and No.53 were used for paper chromatography of porphyrin methyl ester and porphyrin carboxylic acid. The solvent system composed of 2,6-lutidine-water-ammonium hydroxide (10 : 7 : trace) was used for the paper chromatography of porphyrin carboxylic acid. The paper chromatography of porphyrin methyl ester was carried out as

follows; methyl ester on a filter paper was developed with kerosene-chloroform (4.0 : 2.6) (first run), being dried in the dark at room temperature, and then developed with kerosene-n-propanol (5 : 1)(second run) in the same direction. Porphyrins on the filter paper were detected by red fluorescence under ultra-violet light.

2.8. Estimation of porphyrin content

The culture filtrate was extracted with a mixture of acetic acid and ethylacetate (1 : 3). Resulting precipitates were filtered off and thoroughly washed with the same solvent mixture. The filtrate and washings were combined, then washed with deionized water to remove acetic acid. The porphyrin contained in the ethylacetate layer was completely extracted with 5 % HCl. The amount of the porphyrin in the HCl extract, which was identified to be coproporphyrin III as shown later, was assayed by measuring the optical density of Soret band with Shimadzu QB 50 Spectrophotometer.

For estimation of coproporphyrin content, the extinction coefficient given by Falk[13] and the following formula were used.

$$\left\{2 \times OD_{401\ m\mu} - (OD_{430\ m\mu} + OD_{380\ m\mu})\right\} \times 837 \times v/V$$

$$= \text{Coproporphyrin } (\mu g/l)$$

Where,

v = volume of HCl-extract (ml)

V = volume of crude solution (ml).

3. Results

3.1. Characterization of porphyrin produced by Mycobacterium smegmatis

The porphyrins produced by Mycobacterium smegmatis in the culture filtrate and in the cells were extracted with acetic acid-ethylacetate, and characterized by paper chromatography and absorption spectroscopy according to the methods reviewed by Falk[12, 13].

The red pigment extracted from the culture filtrate contained only one component, but the pigment from the bacterial cells consisted of two components, the major component of which was concluded to be the same as that from the culture filtrate in their chromatographic and spectroscopic behaviors. The crude extracts had the Soret band about 400 mμ in their ethylacetate solution, and showed the R_f values of 0.55 (major one) and 0.64 (minor one) on the paper chromatogram developed with 2,6-lutidine-water-ammonium hydroxide system and detected by red fluorescence under ultra-violet light.

These results suggested that the major pigment was a porphyrin having four carboxyl groups and not having any metal, and that the minor one would be a metal-free porphyrin having three or four carboxyl groups[12]. Most common porphyrin having four carboxyl groups is coproporphyrin.

The methyl ester of the major pigment showed the absorption maxima in its chloroform solution at 400.5 (Soret band), 498 (band IV), 532 (band III), 565 (band II), 593 (band Ia), and 620 mμ (band I), as shown in Fig. 1. These absorption maxima were in good agreement with those of coproporphyrin[13], 400, 498, 532, 566, 594, and 621 mμ. The relative absorption intensity at the maxima was also in nearly equal to that of coproporphyrin, as shown in Table 1. On the paper chromatography Mycobacterium porphyrin methyl ester showed the same behavior as authentic coproporphyrin III and did not separate from the authentic one on the co-chromatography as seen in Fig. 2. From these results, we confirmed that the major pigment produced by Mycobacterium smegmatis was coproporphyrin. In order to determine the exact structure of this porphyrin, the IR-spectrum of this pigment was measured in KBr tablet (Fig. 3). The spectrum of this pigment also sustained the above-mentioned structure, but no information was obtained concerning the positions of

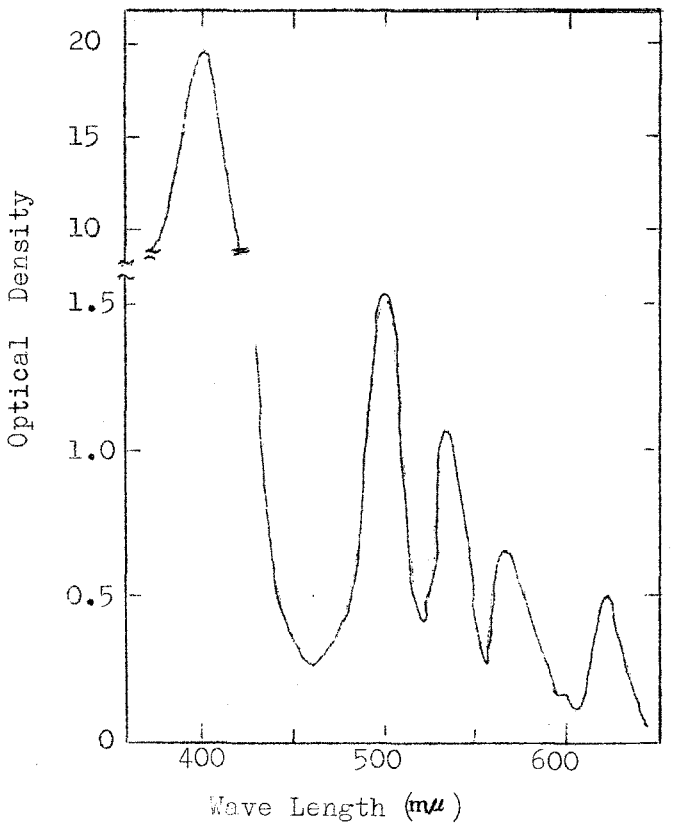


Fig. 1. Visible absorption spectrum of methyl ester of major pigment produced by Mycobacterium smegmatis (in $CHCl_3$)

Table 1. Absorption maxima and relative absorption intensity of porphyrins isolated and those of authentic ones (in $CHCl_3$).[13]

Porphyrin methyl ester	Band					
	Soret	IV	III	II	Ia	I
Major pigment	400.5 mμ (39.2)	498 (3.04)	532 (2.09)	565 (1.43)	593 (0.25)	620 (1.00)
Minor pigment	403 mμ (-----)	500 (4)	534 (12)	569 (15)	---	622 (1)
Uroporphyrin I and III	406 mμ (51.4)	502 (3.78)	536 (2.24)	572 (1.64)	---	627 (1.00)
Coproporphyrin I and III	400 mμ (36.0)	498 (2.87)	532 (1.98)	566 (1.43)	593 (0.29)	621 (1.00)
Protoporphyrin IX	407 mμ (31.8)	505 (2.63)	541 (2.16)	575 (1.38)	603 (0.38)	630 (1.00)

The relative absorption intensity was shown in parentheses, where the intensity of band I was used as the standard.

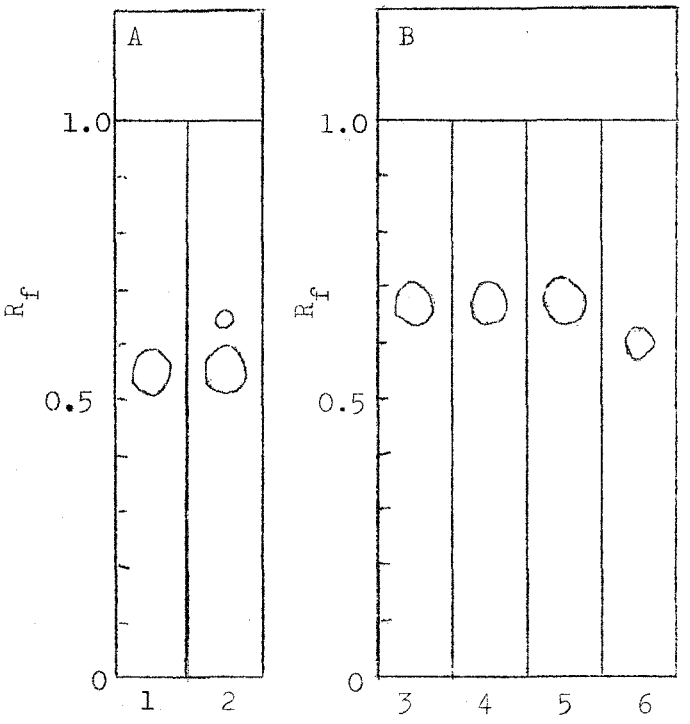


Fig. 2. Paper chromatography of porphyrins

A. Porphyrin carboxylic acid; solvent : 2,6-lutidine-water-ammonium hydroxide (10:7:trace)

1: pigment from culture filtrate

2: pigments from bacterial cells

B. Porphyrin methyl ester; solvent : kerosene-chloroform (4.0:2.6) (first run) and kerosene-n-propanol (5:1) (second run)

3: major pigment, 4: authentic coproporphyrin III,

5: major pigment + coproporphyrin III, 6: minor pigment.

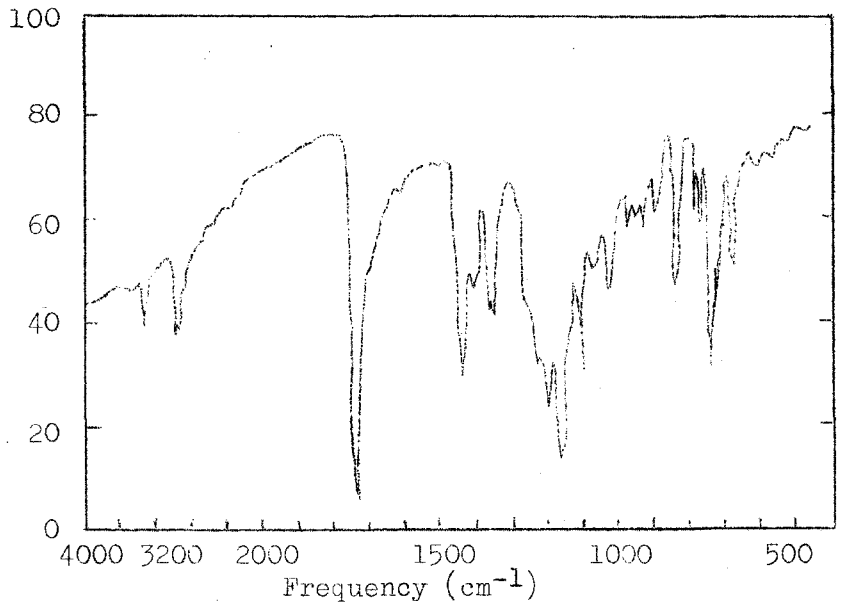


Fig. 3. IR-spectrum of methyl ester of major pigment produced by Mycobacterium smegmatis (KBr tablet).

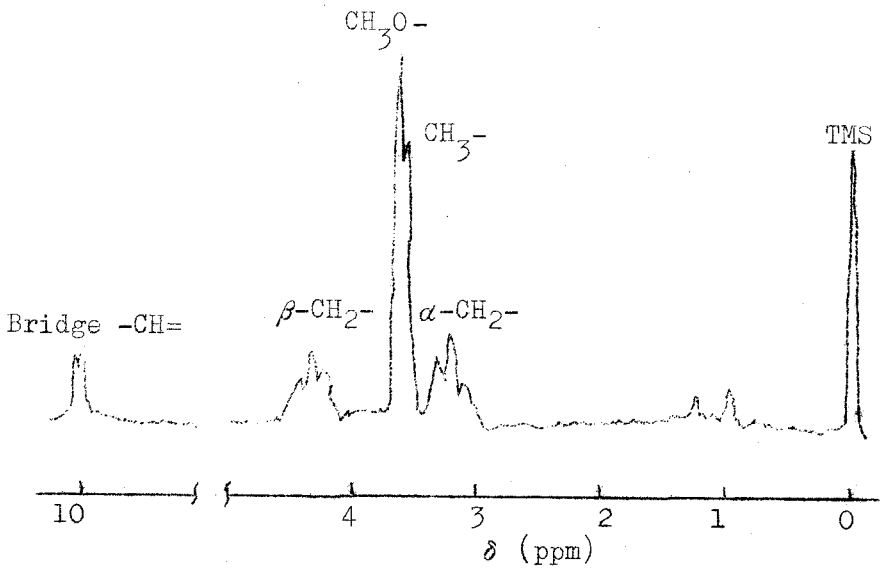


Fig. 4. NMR-spectrum of methyl ester of major pigment produced by Mycobacterium smegmatis (in $CDCl_3$).

methyl- and β -carboxyethyl groups. The result of NMR spectrophotometry finally demonstrated the identity of the porphyrin methyl ester isolated with coproporphyrin III methyl ester. Its characteristic peaks (Fig. 4) were quite the same as those of authentic coproporphyrin III methyl ester and of the literature[14]. Namely, the methine peak (δ = 10 ppm) and methyl peak (δ = about 3.6 ppm) split in the spectrum of coproporphyrin III methyl ester but not in that of coproporphyrin I.

From these results, we concluded that all porphyrin excreted into the culture filtrate and most of porphyrin accumulated in the bacterial cells were metal-free coproporphyrin III.

The minor porphyrin extracted from cells showed the absorption maxima at 403, 500, 569, and 622 mμ in its chloroform solution (Fig. 5). The relative intensity of the absorption maxima differed from that of the known metal-free porphyrins (Table 1), that is, the absorption intensity of band II was largest. This pigment seemed to be metal-free porphyrin, since it had the Soret band at about 400 mμ in its visible absorption spectrum, and showed a red fluorescence under ultra-violet light. We could not determine the structure of this porphyrin, since it was accumulated in only small amounts.

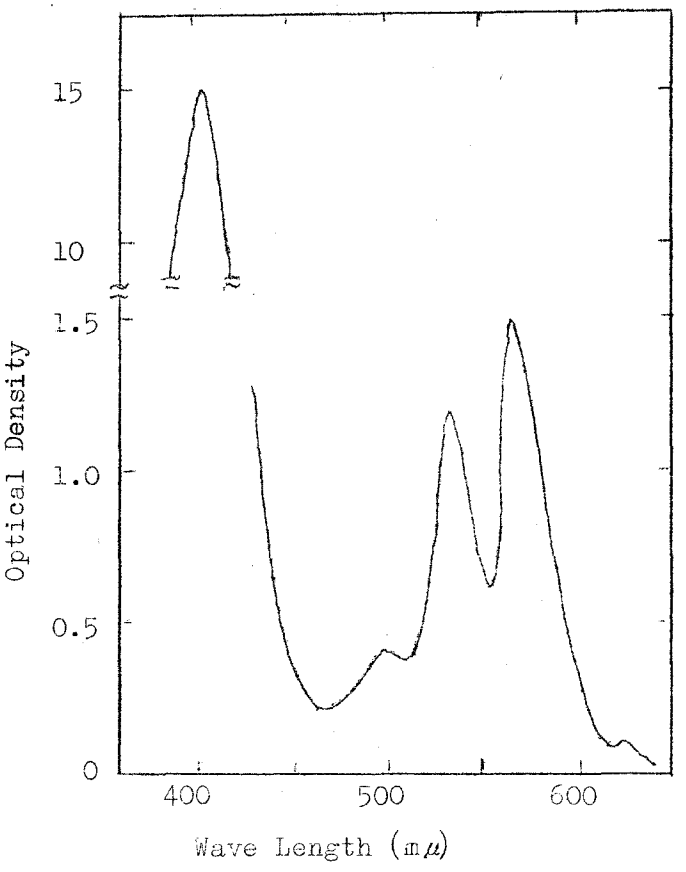


Fig. 5. Visible absorption spectrum of methyl ester of minor pigment extracted from Mycobacterial cells (in $CHCl_3$).

3.2. Excretion of porphyrin by Mycobacterium smegmatis

The porphyrin excretion was about three times larger in the hydrocarbon medium than that in the glucose medium, as shown in Fig. 6. The porphyrin was accumulated at the exponential growth phase in both media. In the hydrocarbon medium, the excretion of porphyrin was only observed under a relatively small aeration, but the cell growth and porphyrin accumulation were not seen under static conditions. The bacterial growth was greatly reduced under the condition which was favorable to the porphyrin excretion (Table 2).

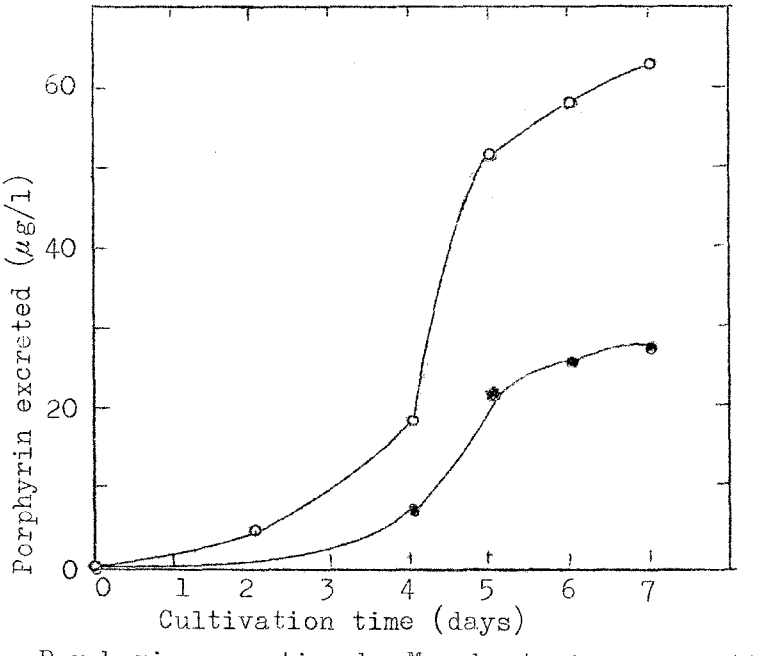


Fig. 6. Porphyrin excretion by Mycobacterium smegmatis.

o---o : hydrocarbon medium

●---● : glucose medium

Table 2. Porphyrin accumulation in culture filtrate by Mycobacterium smegmatis during hydrocarbon assimilation

Fermentor	Volume of medium in fermentor	Aeration	Agitation	K_{La} (hr^{-1})	Growth	Porphyrin accumulation
500-ml shaking	50 ml	None	Rotary	149	++	-
flask	100		shaker	66	++	-
	150		(220 rpm)	49	++	-
	200			36	++	-
	250			28	+	-
10-l glass fermentor	6 l	None	Vibro-mixer	5	+	+
10-l jar	5 l	10 l/min	400 rpm	--	++	-
fermentor	5 l	2 l/min	400 rpm	--	+	-

Porphyrin accumulated was extracted and estimated as mentioned in the text.

The bacterium was cultured for 6 to 8 days at 30°C.

4. Discussion

The results obtained showed that coproporphyrin III was accumulated in the culture filtrate by Mycobacterium smegmatis IFO 3080 under a low aeration in the hydrocarbon medium used.

Both porphyrin and corrinoid are known to be formed via the same biosynthetic route at the early stage, and furthermore, the structure of corrin nucleus is analogous to that of porphyrin, as seen in Fig. 7 and 8.

The relation between the vitamin B_{12} production and the coproporphyrin accumulation was discussed by Fukui et al.[8] when Streptomyces olivaceus was cultured in the various media. That is, they described that the porphyrin accumulation was reduced under the cultural conditions which were favorable to the corrinoid formation. On the other hand, the vitamin B_{12} production by Mycobacterium smegmatis was larger in the hydrocarbon medium than in glucose medium[9] as the case of porphyrin excretion.

Although the precise branching point has not been clarified yet, attentions of many workers[15,16,17] are objected to the relationship between the biosyhthetic pathways of these compounds. The accumulation of coproporphyrin III observed in this study may be explained if δ-aminolevulinic acid synthetase activity is seriously activated and coprogenase activity is

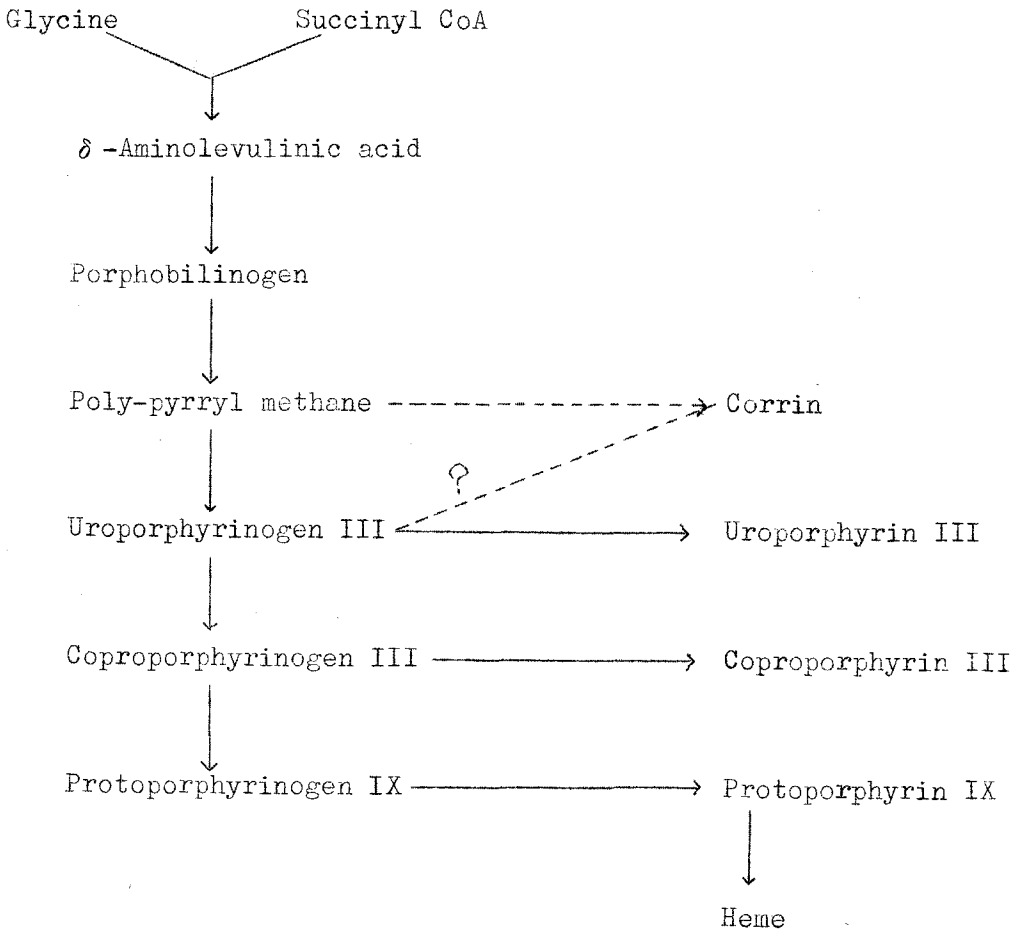
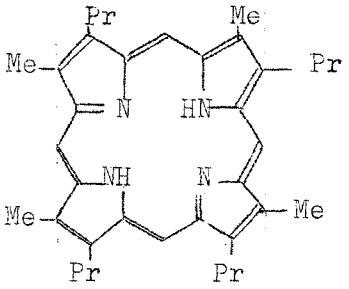
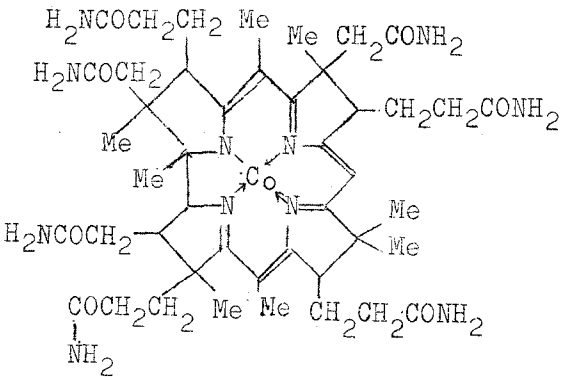


Fig. 7. Biosynthetic pathways of corrinoids and porphyrins



Coproporphyrin III



Corrin nucleus

Fig. 8. Comparison of structures of corrinoid and coproporphyrin III.

markedly decreased, in the hydrocarbon medium under the low oxygen tension.

5. Summary

During the course of the studies on the production of vitamin B_{12} and carotenoids by Mycobacterium smegmatis, we observed that this bacterium accumulated a large amount of red pigment in the culture filtrate under the reduced aeration rather unfavorable to the cell growth and the carotenoid production in hydrocarbon fermentation. The pigment extracted from the culture filtrate and from the bacterial cells with acetic acid-ethylacetate (1 : 3) were characterized as metal-free porphyrins from their visible absorption spectra. Identification of methyl esters of these porphyrins was carried out by paper chromatography, and visible absorption-, infrared-, and nuclear magnetic resonance spectroscopy. The red pigment extracted from the culture filtrate contained coproporphyrin III only, but the red pigment from the bacterial cells contained a small amount of unidentified porphyrin-like substance other than coproporphyrin III. The accumulation of porphyrin was observed under the reduced aeration (K_{La} = 5 hr^{-1}), but not under the larger aeration (K_{La} = above 28 hr^{-1}).

6. References

1). J. Lascelles: Biochem. J., 62, 78 (1956).

2). P.M. Tawnsley and J.B. Neilands: J. Biol. Chem., 224, 695 (1957).

3). R.E. Heady, N.J. Jacobs and R.H. Deibel: Nature, 203, 1286 (1964).

4). H. Chantrenne and C. Courtois: Biochim. Biophys. Acta, 14, 397 (1954).

5). P.J. Dubash and D.V. Rege: Biochim. Biophys. Acta, 141, 209 (1967).

6). P. Schaeffer: Biochim. Biophys. Acta, 9, 261 (1952).

7). V. Musilek: Science, 137, 674 (1962).

8). K. Sato, S. Shimizu and S. Fukui: Agr. Biol. Chem., 32, 1 (1968).

9). K. Fujii, S. Shimizu and S. Fukui: J. Ferment. Technol., 44, 185 (1966).

10). A. Tanaka, T. Nagasaki, M. Inagawa and S. Fukui: J. Ferment. Technol., 46, 468 (1968).

11). A. Tanaka and S. Fukui: J. Ferment. Technol., 46, 214 (1968).

12). J.E. Falk: J. Chromato., 5, 277 (1961).

13). J.E. Falk: Porphyrins and metalloporphyrins (Elsevier Publishing Co., Amsterdam) (1964).

14). E.D. Becker, R.B. Bradley and C.J. Watson: J. Am. Chem. Soc., 83, 3743 (1961).

15). K. Bray and D. Shemin: J. Biol. Chem., 238, 1501 (1963).

16). R.J. Porra: Biochim. Biophys. Acta, 107, 176 (1965).

17). B.F. Burnham and R.A. Plane: Biochem. J., 98, 13C (1966).

Part V. Substrate specificity of yeast in hydrocarbon fermentation

1. Introduction

It is well known that yeast can assimilate n-alkanes, n-alkenes, and hydrocarbon mixtures. Although relative utilizability of n-alkanes by yeast differed among investigators, it is likely that n-alkanes of medium carbon-chain length (C_{12-18}) were most appropriate for yeast[1,2,3]. In the course of our investigation to produce the yeast cell mass and useful cell materials in a hydrocarbon fermentation, we found out that Candida albicans assimilated more readily a hydrocarbon mixture rich in n-undecane than a mixture rich in n-tridecane. Based on this fact, we investigated the substrate specificity of several kinds of yeast in the hydrocarbon fermentation, and found out that Candida albicans and Candida tropicalis showed a difference between the utilizability of the two hydrocarbon mixtures. The strain YH 101-C1, newly isolated by us from garden soil, utilized both of them in a similar degree, though the assimilating rate at the initial growth stage were different. Candida albicans, which could assimilate n-hexadecane and n-octadecane but not kerosene or light oil[4,5], could utilize n-decane, n-undecane, and n-dodecane more readily than longer

carbon-chain n-alkanes. But the organism could not utilize shorter carbon-chain n-alkanes, such as n-hexane and n-octane. In this part, we will describe the substrate specificity of yeast and the relative utilizability of n-alkanes in the hydrocarbon fermentation.

2. Experimental procedure

2.1. Microorganism

The organisms used in this study were as follows;

Candida albicans Berkhout IFO 0583

Candida intermedia NRRL Y-6328-1

Candida tropicalis (Castellani) Berkhout PK-233

Candida tropicalis IFO 0589

YH 101-C-1 (isolated from soil)

These organisms were maintained on malt extract-agar slants.

2.2. Cultivation method

The cultivation method was the same as that shown in Part II. The hydrocarbon medium used was shown in Table 1.

2.3. Determination of cell yield

Determination of the cell yield was carried out as follows; 1 ml of the cultured broth was centrifuged, the cells sedimented were washed with n-hexane and water successively, suspended into 25 ml of water, and then measured by optical density at 570 mμ. The amount of dry cells was determined as follows; 50 ml of broth was centrifuged at 1000 X g for 10 min, the cells sedimented were washed with n-hexane and water successively, and then dried at 105-110°C for 2 hrs. The relation between optical density and dry cell yield were as follows;

C. albicans;	1 g dry cells/l	=1.55 of	optical density at 570 mμ
C. intermedia;	1 "	=3.49	"
C. tropicalis Pk-233;	1 "	=3.14	"
C. tropicalis IFO 0589;	1 "	=2.46	"
YH 101-C-1;	1 "	=3.31	"

Table 1. Composition of hydrocarbon-medium tested.

NH_4NO_3	5.0 g
KH_2PO_4	2.5 g
$MgSO_4 \cdot 7H_2O$	1.0 g
$FeCl_3 \cdot 6H_2O$	0.02g
Corn steep liquor	1.0 g
Tween 85	0.2 ml
Hydrocarbon	5.0—40.0 ml
Tap water	1000 ml
pH	5.2—5.4

2.4. Hydrocarbons

Pure n-alkanes were obtained commercially. "Hydrocarbon mixtures 1 and 2" were the gifts from Dr. M. Shigeyasu, Maruzen Petroleum Industries Co., and "hydrocarbon mixture 3" was a equal volume mixture of "hydrocarbon mixtures 1 and 2". n-Alkane compositions of these three mixtures were as follows;

mixture 1:	n-undecane (n-C_{11})	2.3% by wt.
	n-dodecane (n-C_{12})	13.4%
	n-tridecane(n-C_{13})	51.3%
	n-tetradecane(n-C_{14})	33.0%
mixture 2:	n-decane (n-C_{10})	22.2% by wt.
	n-undecane	44.5%
	n-dodecane	29.1%
	n-tridecane	4.2%
mixture 3:	n-decane	11.1% by wt.
	n-undecane	23.4%
	n-dodecane	21.2%
	n-tridecane	27.8%
	n-tetradecane	16.5%

2.5. Analysis of n-alkanes

Analysis of n-alkanes was carried out by gas chromatography on a column of 5% SE-30 on Celite 545, 80 to 100-mesh, packed in a 1.0-meter stainless-steel column with an internal diameter of 0.3 cm. The apparatus used was Yanagimoto Gas Chromatography GCG 500-T. The amounts of n-alkanes were calculated from the area of the peaks on the charts.

n-Alkanes were extracted with n-hexane from a cultured

broth. The n-hexane layer was dried on sodium sulfate overnight and concentrated to an appropriate volume under a reduced pressure at room temperature (15-30°C) and analyzed by gas chromatography as described above. The recovery test of each n-alkane using this treatment showed 95-101 % of recovery.

3. Results

3.1. Growth of yeast on the "hydrocarbon mixtures 1, 2, and 3"

3.1.1. Candida albicans IFO 0583

As shown in Fig. 1, at the substrate concentration of 0.5 %, Candida albicans showed nearly equal cell yields on the three mixtures, but a highest growth rate was obtained on "mixture 2". Using a higher substrate concentration, the cell yield and the growth rate of Candida albicans on "mixture 1" were smaller than those on the "mixtures 2 and 3", respectively. The "mixture 3" was assimilated in the same degree as the "mixture 2" until at the concentration of 2.0 %, but at 4.0 %, the cell yield on the "mixture 3" was inferior to that on the "mixture 2", although the growth rate of this yeast on both mixtures were almost equal during the initial phase of the growth (Fig. 2). These results suggested that Candida albicans

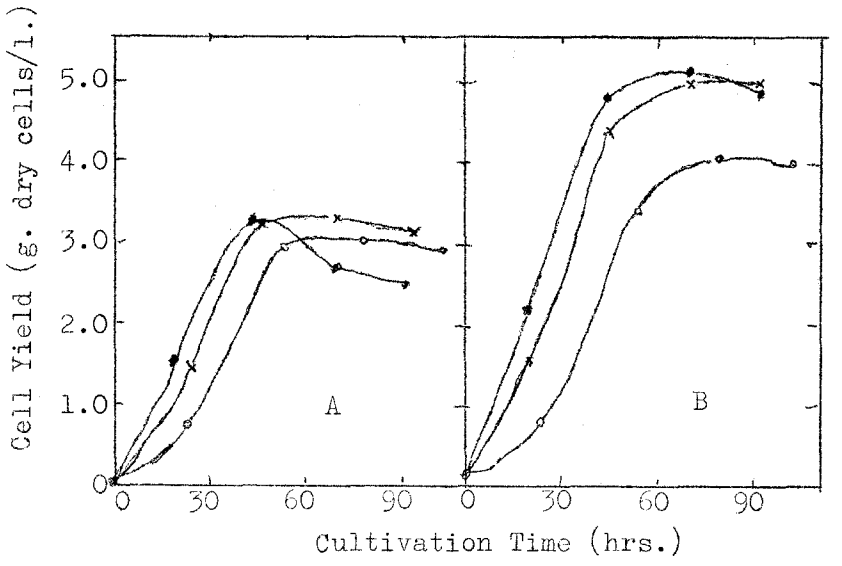


Fig. 1. Typical growth curves of Candida albicans on the three kinds of hydrocarbon mixtures.
"mixture 1"(o— o) "mixture 2" (●— ●) "mixture 3" (×——×)
A: Substrate concentration of 0.5 v/v%
B: Substrate concentration of 1.0 v/v%
Cultivation was carried out on a rotary shaker (220 rpm) at 30°C.

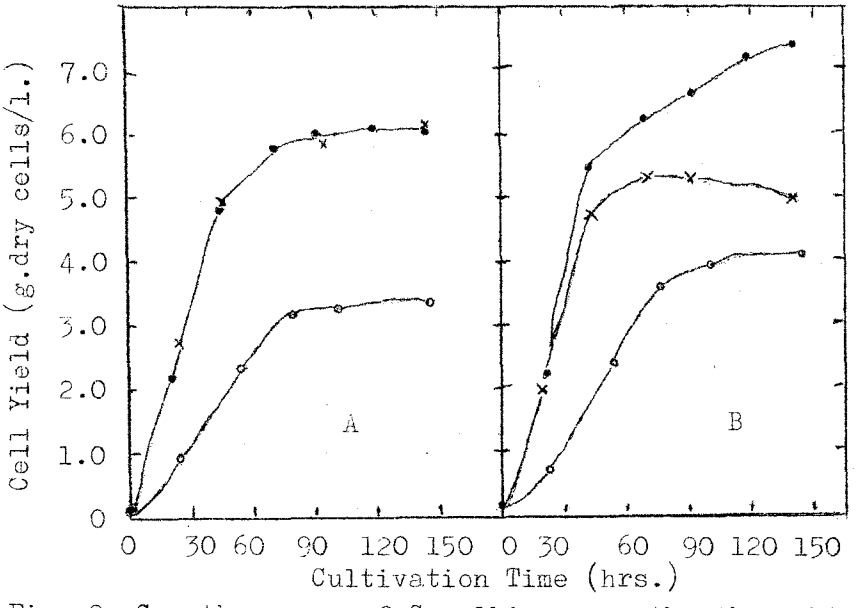


Fig. 2. Growth curves of C. albicans on the three kinds of hydrocarbon mixtures.
"mixture 1" (o— o) "mixture 2" (●— ●) "mixture 3" (×— ×)
A: Substrate concentration of 2.0 v/v%
B: Substrate concentration of 4.0 v/v%

could assimilate n-undecane more readily than n-tridecane.

3.1.2. Candida intermedia NRRL Y-6328-1

This yeast did not grow well on the hydrocarbon mixtures and n-hexadecane compared with other yeast used in this study. The cell yield of this yeast on the "mixture 1" was lower than those on the "mixtures 2 and 3" (Table 2 and Fig. 3).

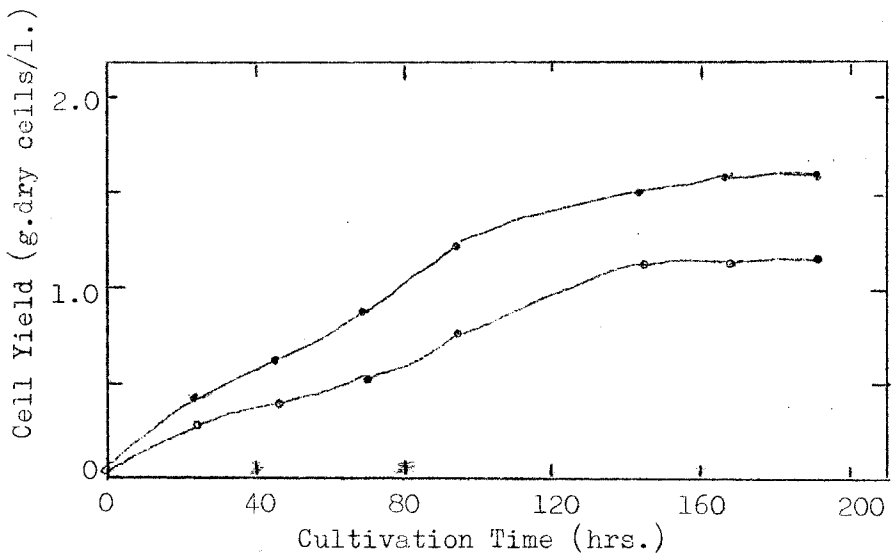


Fig. 3. Typical growth curves of C. intermedia on the hydrocarbon mixtures.
"mixture 1" (o—o) "mixture 2" (●—●)
Substrate concentration was 1.0 v/v%.
Cultivation was carried out on a rotary shaker (220 rpm) at 30°C.

3.1.3. Candida tropicalis IFO 0589

This yeast grew well on the "mixtures 1 and 2" as well as on n-hexadecane. The cell yield and the growth rate of this yeast on the "mixture 1" were similar to those on the "mixture 2" (Fig. 4A).

3.1.4. Candida tropicalis PK-233

No significant differences were observed among the initial growth rates of this yeast on the three mixtures mentioned above, but the final cell yield on the "mixture 1" was relatively small. This tendency was more obvious at the substrate concentrations of 2.0 % (Table 2 and Fig. 4B).

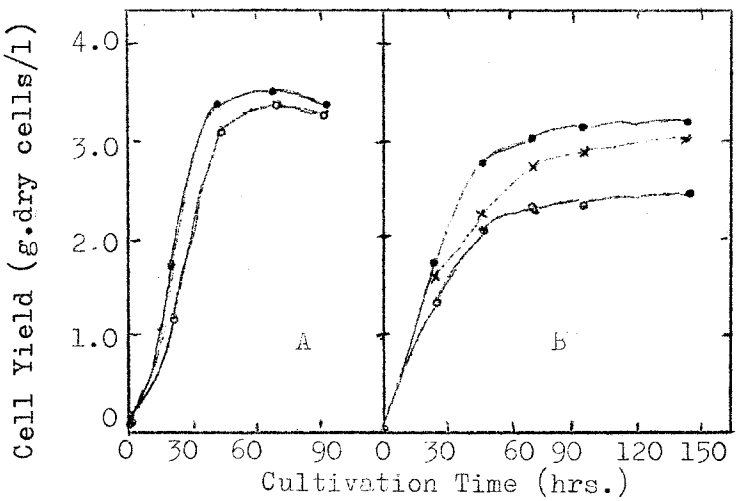


Fig. 4. Typical growth curves of C. tropicalis on the three kinds of hydrocarbon mixtures.
"mixture 1" (o—o) "mixture 2" (●——●) "mixture 3" (×——×)
A: C. tropicalis IFO 0589 B: C. tropicalis Pk-233
Cultivation was carried out on a rotary shaker (220 rpm) at 30°C using 1.0 v/v% of substrate.

3.1.5. YH 101-C-1

The final cell yields on the "mixtures 1 and 2" were nearly same (Table 2), although the initial growth rates on these two mixtures were fairly different. At the early cultivation period after inoculation, this yeast grew well on the "mixture 2" but scarcely on the "mixture 1" (Fig. 5). After this induction period, this yeast grew well on the "mixture 1" with the growth rate comparable to that on the "mixture 2". But the fermentation period when the cell yield reached to the maximum was postponed on the "mixture 1" about 25 hrs compared with that on the "mixture 2". The final cell yields were 4.7 g dry cells per liter on the "mixture 1" and 4.55 g on the "mixture 2", respectively, and little difference was seen between the cell yields on the two hydrocarbon mixtures.

3.2. Growth of yeast on pure n-alkanes

As described above, Candida albicans as well as the other yeast showed the substrate specificity for the n-alkane mixtures. Then, we investigated the substrate specificity for pure n-alkanes using Candida albicans. Candida albicans could grow on n-alkanes of C_{10-20}, but could not on n-hexane and n-octane. Among these oxidizable n-alkanes, n-alkanes

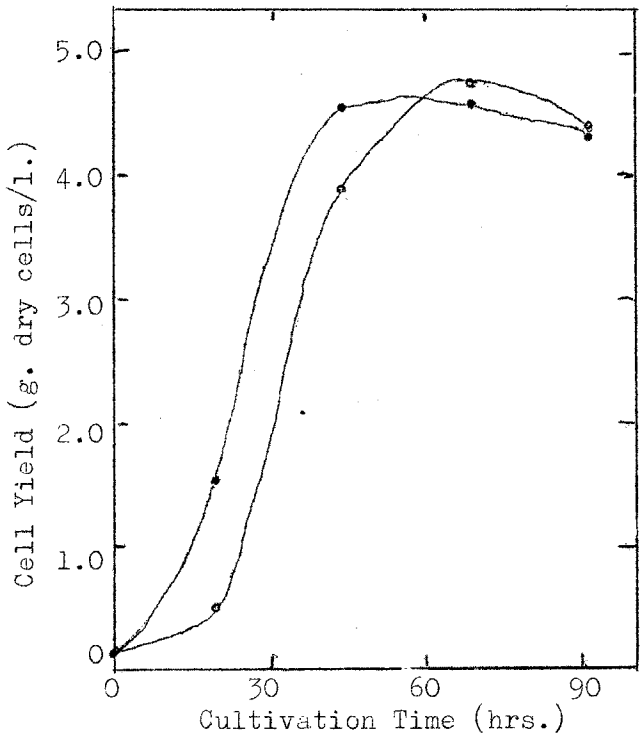


Fig.5. Typical growth curves of YH101-C1 on the hydrocarbon mixtures.
"mixture 1" (o—o) "mixture 2" (●—●)
Cultivation method was same as that shown in Fig. 4.

Table.2. Yeast cell yield on three hydrocarbon-mixtures and n-hexadecane.

H.C. mixture	Concn. (v/v%)	Maximum cell yield (g dry cells/l)				
		C. albicans	C. intermedia	C.tropicalis 0589	C.tropicalis PK-233	YH101 -C1
1	0.5	3.08	-	-	-	-
	1.0	4.04	1.17	3.35	2.47	4.47
	2.0	3.38	-	-	2.30	-
	4.0	4.10	-	-	-	-
2	0.5	3.31	-	-	-	-
	1.0	5.10	1.60	3.53	3.15	4.55
	2.0	6.06	-	-	3.55	-
	4.0	7.40	-	-	-	-
3	0.5	3.31	-	-	-	-
	1.0	4.95	1.73	-	2.99	-
	2.0	5.15	-	-	3.02	-
	4.0	5.31	-	-	-	-
n-Hexadecane	1.0	4.40	-	2.63	3.04	4.40

Cultivation was carried out on a rotary shaker (220 rpm) at 30°C for 6 days.

with relative shorter carbon chains (C_{10-12}) were the best substrates for this yeast and n-hexadecane was next to these. On the contrary, n-tridecane, n-tetradecane and n-pentadecane were the poor substrates for this yeast. This result was in accord with the facts that Candida albicans could grow better on the "hydrocarbon mixture 2" rich in n-undecane than the "mixture 1" rich in n-tridecane as mentioned above. Therefore, we concluded that the difference among the utilizability of

Table 3. Growth of C. albicans on various n-alkanes.

	Cell yield(g dry cells/l)	
	2 day culture	4 day culture
None	0.28	0.24
n-Hexane	0.27	0.24
n-Octane	0.31	0.32
n-Decane	5.65	5.68
n-Undecane	5.13	5.12
n-Dodecane	5.48	5.45
n-Tridecane	2.78	3.67
n-Tetradecane	3.00	3.42
n-Pentadecane	2.07	2.82
n-Hexadecane	4.17	4.40
n-Heptadecane	3.83	3.88
n-Octadecane	3.24	3.89
n-Nonadecane	3.78	3.94
n-Eicosane	3.31	3.31

Cultivation was carried out on a rotary shaker (220 rpm) at 30°C.
Each n-alkane was added at the concentration of 1.0 v/v%.

the three hydrocarbon mixtures by the yeast resulted from its different utilizability of pure n-alkanes. We could not detect the difference between the oxidizability of the even carbon number alkanes and the odd carbon number alkanes (Table 3).

Candida tropicalis IFO 0589, Candida tropicalis PK-233 and YH 101-C-1 grew well using n-hexadecane, but Candida intermedia could not grow well on this alkane (Table 2).

3.3. Relative utilizability of n-alkanes

As described above, the growth rate and the maximal cell yield of Candida albicans were excellent when used n-alkanes of carbon number 10 to 12 as carbon sources, while n-alkanes of C_{13-15} were not good substrates. This result was in accord with the fact that the "hydrocarbon mixture 2" including n-decane (22.2 % by weight), n-undecane (44.5 %), n-dodecane (29.1 %) and n-tridecane (4.2 %) was the better substrate for Candida albicans than the "mixture 1" including n-undecane (2.3 %), n-dodecane (13.4 %), n-tridecane (51.3 %) and n-tetradecane (33.0 %). In relation to these results, we investigated the relative utilizability of n-alkanes in the hydrocarbon mixture using gas chromatographic technique. Candida albicans growing on the "mixture 1" revealed a relatively small consumption rate of substrate (Table 4). After 4

days' cultivation, only thirty per cent of substrate was consumed, and even n-undecane and n-dodecane remained in thirty-seven and fifty-seven per cent of the initial amounts, respectively. Consequently, the growth rate and cell yield of this yeast were small on this substrate. On the contrary, Candida albicans readily assimilated the "mixture 2" (Table 5). In this case, the consumption rates of n-decane and n-undecane were especially large, but that of n-tridecane was relatively small. After 4 days' cultivation, nearly all of substrate was utilized. The typical gas chromatographic patterns given in Fig. 6 shows the utilization of this substrate by Candida albicans until 4 days' cultivation. On the other hand, Candida albicans growing on the "mixture 3" (equal volume mixture of the "mixtures 1 and 2") exhibited relatively small growth rate and small consumption rate compared with those on the "mixture 2", although this yeast gave the same cell yields on both the substrates. In this case, n-decane and n-undecane were also assimilated more readily than n-tridecane or n-tetradecane. After 4 days' cultivation when about ninety per cent of the substrate was utilized, nearly all of n-decane and n-undecane were assimilated, whereas about twenty per cent of n-tridecane and n-tetradecane remained (Table 6).

From these results, we concluded that Candida albicans

Table 4. Consumption of "hydrocarbon mixture 1" by C. albicans.

Cultivation time (hr)	Cell yield (g dry cells/l)	Residual n-alkanes									
		n-C_{11}		n-C_{12}		n-C_{13}		n-C_{14}		Total	
		mg/100ml	%	mg/100ml	%	mg/100ml	%	mg/100ml	%	mg/100ml	%
0	0.10	35	100	202	100	771	100	496	100	1504	100
22	1.02	26	74	155	77	646	84	427	86	1254	83
48	2.35	21	60	136	67	588	76	390	79	1135	76
70	3.03	14	40	119	59	541	70	374	75	1048	70
94	2.98	13	37	116	57	532	69	370	75	1031	69

Cultivation was carried out on a rotary shaker (220 rpm) at 30°C. Hydrocarbon mixture was added at the concentration of 2.0 v/v%, and estimated gas-chromatographically.

Table 5. Consumption of "hydrocarbon mixture 2" by C. albicans.

Cultivation time (hr)	Cell yield (g dry cells/l)	Residual n-alkanes									
		n-C_{10}		n-C_{11}		n-C_{12}		n-C_{13}		Total	
		mg/100ml	%	mg/100ml	%	mg/100ml	%	mg/100ml	%	mg/100ml	%
0	0.13	330	100	661	100	432	100	62	100	1468	100
24	2.98	201	61	465	70	336	78	49	79	1051	71
48	5.20	58	18	172	26	168	39	26	42	424	29
72	5.85	15	5	47	7	68	16	14	23	144	10
96	5.85	0	0	5	1	11	3	2	3	18	1

Cultivation and estimation methods were same as those shown in Table 4.

Table 6. Consumption of "hydrocarbon mixture 3" by C. albicans.

Cultivation time (hr)	Cell yield (g dry cells/l)	Residual n-alkanes									
		n-C_{10}		n-C_{11}		n-C_{12}		n-C_{13}		n-C_{14}		Total
		mg/100ml	%	mg/100ml	%	mg/100ml	%	mg/100ml	%	mg/100ml	%	mg/100ml
0	0.11	165	100	348	100	317	100	417	100	248	100	1495
46	4.47	41	25	111	32	137	43	205	49	124	50	618
70	5.15	4	2	17	5	38	12	81	19	53	21	193
94	5.91	2	1	10	3	30	10	78	19	44	18	164

Cultivation and estimation methods were same as those shown in Table 4.

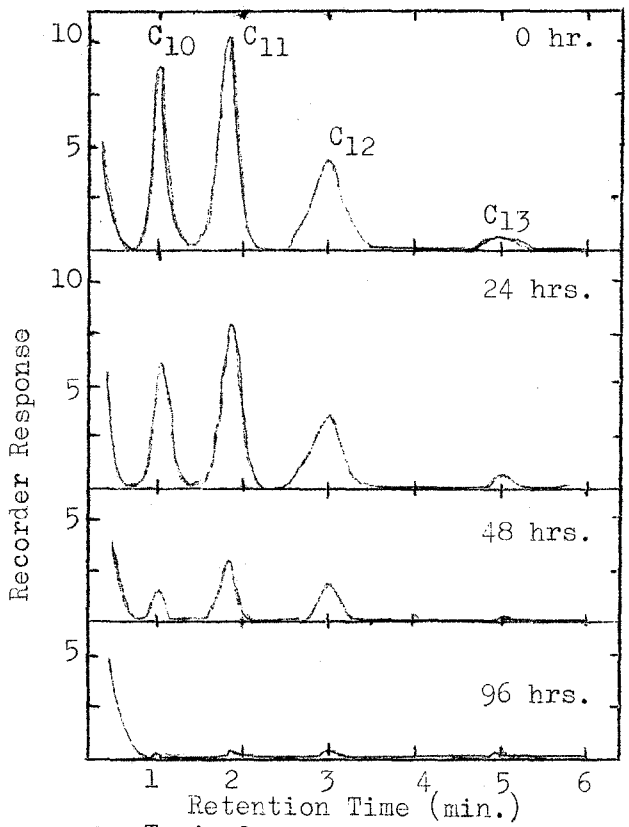


Fig.6. Typical gas chromatographic patterns of residual substrates.

C. albicans was cultivated on "hydrocarbon mixture 2" (2.0 v/v %), using a rotary shaker (220 rpm). Residual substrates were determined at 0 (before inoculation), 24, 48 and 96 hrs of cultivation.

could more readily utilize n-decane and n-undecane than n-tridecane and n-tetradecane, and that a larger amount of the latter alkanes presented in a hydrocarbon mixture would inhibit the assimilation of the former alkanes by this yeast competitively. Consequently it would be concluded that, among n-alkanes of carbon number 10 to 14, the shorter the carbon chain of the alkane was, the larger the relative utilizability by Candida albicans was.

The similar results were obtained using Candida tropicalis PK-233 (Table 7, 8 and 9). In this case, n-decane and n-undecane were better substrate than n-tridecane and n-tetradecane for this yeast, too. But, the inhibitory effect of the latter two alkanes was not seen so obviously as in the case of Candida albicans (Table 7 and 9).

Table 7. Consumption of "hydrocarbon mixture 1" by C. tropicalis Pk-233.

Cultivation time (hr)	Cell yield (g dry cells/l)	Residual n-alkanes									
		n-C_{11}		n-C_{12}		n-C_{13}		n-C_{14}		Total	
		mg/100ml	%	mg/100ml	%	mg/100ml	%	mg/100ml	%	mg/100ml	%
0	0.06	35	100	202	100	771	100	496	100	1504	100
20	1.09	22	63	160	79	623	81	420	85	1225	81
44	2.15	13	37	113	56	438	57	301	61	865	58
70	2.30	7	20	74	37	312	40	200	40	593	39
92	2.19	6	17	63	31	284	37	178	36	531	35

The methods of cultivation of the yeast and of estimation of hydrocarbons were same as those shown in Table 4.

Table 8. Consumption of "hydrocarbon mixture 2" by C. tropicalis Pk-233.

Cultivation time (hr)	Cell yield (g dry cells/l)	Residual n-alkanes									
		n-C_{10}		n-C_{11}		n-C_{12}		n-C_{13}		Total	
		mg/100ml	%	mg/100ml	%	mg/100ml	%	mg/100ml	%	mg/100ml	%
0	0.06	330	100	661	100	432	100	62	100	1486	100
20	1.38	127	38	366	55	291	67	37	60	821	55
44	2.89	44	13	197	30	181	42	23	37	445	30
70	3.55	4	1	87	13	120	28	14	23	225	15
92	3.37	trace	0	26	4	44	10	trace	0	70	5

The methods of cultivation and estimation were same as those shown in Table 4.

Table 9. Consumption of "hydrocarbon mixture 3" by C. tropicalis Pk-233.

Cultivation time (hr)	Cell yield (g dry cells/l)	Residual n-alkanes											
		n-C_{10}		n-C_{11}		n-C_{12}		n-C_{13}		n-C_{14}		Total	
		mg/100ml	%	mg/100ml	%	mg/100ml	%	mg/100ml	%	mg/100ml	%	mg/100ml	%
0	0.06	165	100	348	100	317	100	417	100	248	100	1495	100
20	1.37	77	47	238	68	247	78	351	84	213	85	1126	75
44	2.78	33	20	132	38	173	55	255	61	154	62	747	50
70	3.02	21	13	110	32	146	46	230	55	140	56	647	43
92	2.98	12	7	67	19	114	36	181	43	109	44	483	32

The methods of cultivation and estimation were same as those shown in Table 3.

4. Discussion

Although a number of investigation have been made about the utilization of hydrocarbons by yeast, especially Candida genus[1,2,3,6,7,8] most of these were concerned with pure alkanes. The results from several laboratories carried out under various cultural conditions suggested that most kinds of yeast would exhibit the substrate specificity even if only n-alkanes were used as a sole carbon source, and that n-alkanes of medium carbon chain-length(C_{10-20}) would be the best carbon sources for yeast in hydrocarbon fermentation. Johnson[1] reported that Candida intermedia or a closely related species could assimilate n-alkanes of C_{12}, C_{14}, C_{16}, C_{17} and C_{18}, and that the generation time of this yeast was shortest on n-octadecane. On the other hand, Candida reukaufii could utilize n-decane, n-dodecane and n-tetradecane, but could not n-hexadecane and n-octadecane, as compared with Candida lipolytica and Candida pulcherrima which could assimilate all these n-alkanes[2]. Takeda et al.[3] investigated on the utilizability of pure n-alkanes using several kinds of yeast, and reported that n-alkanes from C_{11} to C_{18} were assimilated well. Our results revealed that Candida albicans could assimilate n-decane, n-undecane and n-dodecane most readily, but n-tridecane, n-tetradecane and n-pentadecane relatively

slowly, and could not utilize n-hexane and n-octane. We have not any experimental data to explain why there exist this specificity among a homologous series of n-alkanes. From the results of the physiological and metabolic studies on a Micrococcus capable of oxidizing hydrocarbons, Harris[9] explained this substrate specificity on the basis of the molecular configuration. That is, he suggested that n-octane and n-dodecane which had the exposed terminal methyl groups on Hirschfelder molecular models, were readily oxidized by this Micrococcus, but that n-decane whose terminal methyl groups were tightly retained in folded form of this molecular, was oxidized somewhat slowly. But these results were not consistent with our results that n-decane was one of the best substrate for Candida albicans. We supposed that the turn over numbers of hydrocarbon-oxidizing enzyme system would vary with various n-alkanes from which the difference in the utilizability of n-alkanes would arise, since the higher concentration of n-tridecane or n-tetradecane was more inhibitory to the utilization of n-decane or n-undecane by Candida albicans (Table 4, 5 and 6). The similar result was obtained using Candida tropicalis PK-233.

From these facts, it will be recommended that, when the "hydrocarbon mixture 1" (rich in n-tridecane) will be used

for cell mass production of Candida, n-decane, n-undecane, or n-dodecane should be added as a supplement.

5. Summary

In the course of the studies on the utilization of hydrocarbons by several kinds of yeast, we found out that Candida albicans, Candida intermedia, Candida tropicalis and YH 101-C-1 (isolated from soil) could grow better on "hydrocarbon mixture 2" (rich in n-undecane) than on "hydrocarbon mixture 1" (rich in n-tridecane). Based on this fact, we investigated the substrate specificity of Candida albicans using pure n-alkanes. Among the n-alkanes tested, n-decane, n-undecane, and n-dodecane were the best substrates, n-hexadecane was next to them, but n-tridecane, n-tetradecane, and n-pentadecane were inferior under the experimental conditions. n-Hexane and n-octane were not utilized by this yeast. In order to examine the relative utilizability of n-alkanes in the hydrocarbon medium containing a mixture of various hydrocarbons, we determined the residual substrates in the cultured broth using gas chromatographic technique. The results obtained showed that the relative utilizability of n-alkanes in the hydrocarbon mixtures by Candida albicans was as follows; n-decane > n-undecane > n-dodecane > n-tridecane > n-tetradecane.

A higher concentration of n-tridecane or n-tetradecane was rather inhibitory for the assimilation of n-decane or n-undecane by Candida albicans. In the case of Candida tropicalis PK-233, similar results were obtained.

6. References

1). M.J. Johnson: Chem. Ind., 36, 1532 (1964).

2). A.J. Markovetz and R.E. Kallio: J. Bacteriol., 87, 968 (1964).

3). I. Takeda, T. Iguchi, T. Kawamura, S. Horiguchi and S. Hayakawa: Agr. Biol. Chem., 29, 796 (1965).

4). A. Tanaka and S. Fukui: J. Ferment. Technol., 45, 611 (1967).

5). A. Tanaka, H. Maki and S. Fukui: J. Ferment. Technol., 45, 1156 (1967).

6). R. Sheda and P. Bos: Nature, 211, 660 (1966).

7). A. Tanaka and S. Fukui: Mizu-Shori Gijutsu, 8 (10), 9 (1967).

8). M.J. Klug and A.J. Markovetz: Appl. Microbiol., 15, 690 (1967).

9). J.O. Harris: Arch. Biochem. Biophys., 70, 457 (1957).

10). I. Tanabe, J. Okada and H. Ono: Agr. Biol. Chem., 30, 1175 (1966).

Part VI. Growth ability of yeasts and molds on hydrocarbons and their derivatives

Chapter 1. Growth of yeasts on hydrocarbons and their derivatives

1. Introduction

Yeast, especially belonging to the genus of Candida, is believed to be one of the most useful organism to produce "single cell protein". The production of yeast cell mass has been attempted using crude oil as carbon source in Europe, while n-alkane fractions derived from crude oil have been used as growth substrates for yeast in Japan. Although n-alkanes of medium chain-length (C_{10-20}) are surely better substrates for various microorganisms than crude oil, it will be disadvantageous to use these alkanes in their pure forms as a starting materials in fermentation process at present because of their high costs. Various yeasts are known to utilize n-alkanes and/or n-alkenes of medium chain-length (C_{10-20}) and these include Candida[1-8], Pichia[9], Debaryomyces[9], Torulopsis[9], Rhodotorula[7,9], Hansenula[7], Mycotorula[8], and Tricosporon[7], and so on. On the other hand, a relatively few yeasts could grow on crude hydrocarbon

mixtures, that is some strains of Candida[3,4,10,11], Rhodotorula[8,10], Hansenula[3], Mycotorula[8] and Brettanomyces[12] on kerosene, and Candida tropicalis on light oil, liquid paraffin and heavy oil[4].

Therefore, it is important problem to search a certain carbon source being available easily and to screen some useful yeasts which can assimilate crude hydrocarbon mixtures. Hence we investigated the growth of several yeast on n-alkanes, their derivatives and crude hydrocarbon mixtures in this chapter.

2. Experimental procedure

2.1. Microorganism

The organisms used in this study were gifted from the Department of Fermentation Technology, Osaka University and the Institute for Fermentation, Osaka, and maintained on malt extract-agar slants.

2.2. Cultivation method

Cultivation method for yeast was the same as that shown in Part I. Carbon sources were added at the concentrations of 1.0 v/v % or 1.0 w/v %.

3. Results and discussion

As shown in Table 1, Candida albicans, Candida lipolytica, Candida tropicalis and YH 101-C-1 (isolated from soil) could assimilate well n-hexadecane and n-octadecane. However, cetyl alcohol, stearyl alcohol, palmitic acid and stearic acid, which were the oxidative intermediates of n-hexadecane and n-octadecane degradation, were not necessarily good substrates for these yeasts except YH 101-C-1. We expected that palmitic acid and stearic acid would be superior substrates even for the yeasts which could not assimilate hydrocarbons, since these acids were the most common fatty acids occurred in these organisms[13,14]. The results showed that these acids could not support the growth of most yeasts tested including hydrocarbon-utilizers. This fact would be ascribed to the permeability of these acids, since the intracellular fatty acids are known to be utilized as carbon sources. Aida et al.[15] demonstrated the inhibitory effect of higher fatty acids on the growth of Mycotorula japonica, a hydrocarbon-utilizing yeast cultured on n-hexadecane. Similar results were also obtained on some bacteria[16]. This suggested that fatty acids would interact with permease or with hydrocarbon-oxidizing enzymes at the cell wall and competitively inhibit the incorporation or the oxidation of hydrocarbons as the

results. The possibility would not be ruled out that some yeasts, such as YH 101-C-1, assimilates the fatty acids via the di-terminal oxidation system demonstrated in Candida rugosa[17]. In conclusion, the derivatives of hydrocarbons so far tested were found out not to be good substrates for yeast growth. The crude hydrocarbon mixtures, such as wax, paraffin (m.p. 42-44°), light oil and kerosene were not also assimilated by the yeasts used in this study.

4. References

1). M.J. Johnson: Chem. Ind., 1964, 1532

2). J. Takahashi, Y. Kawabata and K. Yamada: Amino acid. Nucleic acid, 10, 91 (1964)

3). K. Komagata, T. Nakase and N. Katsuya: J. Gen. Appl. Microbiol., 10, 313 (1964)

4). S. Otsuka, R. Ishii and N. Katsuya: J. Gen. Appl. Microbiol., 12, 1 (1966)

5). I. Takeda: Hakko Kyokai-Shi, 24, 443 (1966)

6). M.J. Klug and A.J. Markovetz: Appl. Microbiol., 15, 690 (1967)

7). A.J. Markovetz and R.E. Kallio: J. Bacteriol., 87, 968 (1964)

8). T. Aida and K. Yamaguchi: Nogei-Kagaku Zasshi, 40,

Yeast \ Carbon source	n-Hexadecane	n-Octadecane	Cetyl alc.	Stearyl alc.	Palmitic a.	Stearic a.	Wax	Paraffin	Light oil	Kerosene
Candida albicans IFO 0583	+++	++	++	±	±	±	±	±	-	-
Candida lipolytica IFO 0717	+++	+	+	+	+	+	-	±	-	-
Candida rugosa IFO 0591	+	+	+	+	+	+	±	+	-	-
Candida tropicalis IFO 0589	++	++	++	+	+	+	-	±	-	-
Candida utilis IFO 0619	±	+	+	+	+	±	±	±	-	-
Debaryomyces kloeckeri IFO 0034	-	-	±	-	+	±	±	-	-	-
Endomyces fibuliger IFO 0103	-	-	-	-	±	-	-	-	-	-
Hansenula anomala IFO 0127	-	-	±	-	±	±	-	-	-	-
Pichia membranaefaciens IFO 0188	±	-	+	-	-	±	-	+	-	-
Rhodotorula rubra IFO 0382	-	±	+	-	+	-	-	-	-	-
Schizosaccharomyces pombe IFO 0346	-	-	-	-	-	±	-	-	-	-
Zygosaccharomyces soya IFO 0495	-	-	-	-	+	+	-	-	-	-
YH 101-C-1 (isolated from soil)	+++	+++	+++	+++	++	++	±	+	±	-

Table 1. Growth of yeasts on hydrocarbons and their derivatives.

Yeasts were cultured on a rotary shaker (220 rpm) for 7 to 9 days.

+++ : abundant growth, ++ : moderate growth, + : slight, visible growth,

± : questionable growth, and - : no growth.

119 (1966)

9). P. Sheda and P. Bos: Nature, 211, 660 (1966)

10). K. Komagata, T. Nakase and N. Katsuya: J. Gen. Appl. Microbiol., 10, 323 (1964)

11). I. Tanabe, J. Okada and H. Ono: Agr. Biol. Chem., 30, 1175 (1966)

12). J. Takahashi, Y. Imada and K. Yamada: Agr. Biol. Chem., 27, 396 (1963)

13). M.J. Klug and A.J. Markovetz: J. Bacteriol., 93, 1847 (1967)

14). M. Mizuno, Y. Shimojima, T. Iguchi, I. Takeda and S. Senoh: Agr. Biol. Chem., 30, 506 (1966)

15). T. Aida, Y. Koh and K. Yamaguchi: Proc. of the Annual Meeting of Agr. Chem. Soc., Japan (Nagoya) (1968)

16). K. Fujii: (Personal communication)

17). H. Iizuka, M. Iida and Y. Unami: J. Gen. Appl. Microbiol., 12, 119 (1966)

Chapter 2. Growth of molds on hydrocarbons

1. Introduction

It is well known that a number of molds including Aspergillus, Penicillium and Fusarium can assimilate various hydrocarbons as reviewed by ZoBell[1] and Davis[2]. Recently Markovetz et al.[3] reported the utilizability of n-alkanes and 1-alkenes by various molds and recognized that Cunninghamella was the most active organism among the molds tested. In this study, they detected a monocarboxylic acid, a primary alcohol, and a secondary alcohol as oxidation products of these hydrocarbons used.

It will be interesting to produce some useful materials, such as fatty substances, organic acids, antibiotics and extracellular enzymes, from hydrocarbons using some strains of molds. In this chapter we dealt with the capabilities of molds to grow on a n-alkane mixture in three different culture conditions: shaking culture, surface culture and interface culture (cultivation at the interface of aqueous layer and hydrocarbon layer). Some molds could grow on a hydrocarbon-mineral salts medium under relatively anaerobic conditions as well as aerobic conditions. The fact seems to offer an interesting problem concerning the power input for agitation

and aeration in hydrocarbon fermentation.

2. Experimental procedure

2.1. Microorganism

The organisms used in this study were obtained from the Department of Fermentation Technology, Osaka University and the Institute for Fermentation, Osaka, and maintained on malt extract-agar slants.

2.2. Cultivation method

For preparing an inoculum, the mold was grown on malt extract-agar slant for 3 to 4 days at 30°C, and a loopful of the cells was transferred into a test tube containing 20 ml of Bushnell-Haas medium (Table 1) and 0.4 ml or 15 ml of a hydrocarbon. In the case of shaking culture, a test tube containing 20 ml of medium and 0.4 ml of a hydrocarbon was shaken by reciprocation (120 strokes per min) at 25-27°C, or a 500-ml flask containing 100 ml of medium and 2.0 ml of a hydrocarbon was shaken by rotation (220 rpm) at 30°C. In the case of surface and interface cultures, test tubes containing 20 ml of medium and 0.4 or 15 ml of hydrocarbon, respectively, were cultured statically at 30°C.

Table 1. Composition of hydrocarbon medium used.

NH_4NO_3	1.0 g
KH_2PO_4	1.0 g
K_2HPO_4	1.0 g
$MgSO_4.7H_2O$	0.2 g
$CaCl_2.2H_2O$	0.02 g
$FeCl_3.6H_2O$	0.05 g
Hydrocarbon	20 ml
Tap water	1000 ml
pH	6.2

2.3. Hydrocarbon

The "hydrocarbon mixture 1" was mainly used as a sole carbon source. This mixture contained n-undecane 2.3 %, n-dodecane 13.4 %, n-tridecane 51.3 % and n-tetradecane 33.0 % by weight.

Other chemicals were obtained commercially.

3. Results and discussion

As shown in Table 2, some strains belonging to the genus of Aspergillus, Fusarium, Penicillium and Cladosporium could grow on the "hydrocarbon mixture 1". As compared with

bacteria and yeast which could grow only under aerobic condition, these molds were able to grow under relatively anaerobic condition (interface culture) as well as aerobic condition (shaking culture). Most of molds, which could assimilate hydrocarbons, showed the maximal growth between 10 to 20 days, but a few showed longer lag times. Molds grown on hydrocarbons under shaking formed submerged hyphae in flasks or aerial hyphae and submerged hyphae in test tubes. On the other hand, molds showed mat-like growth at the interface of air and medium in surface cultures and at interface of aqueous and liquid hydrocarbon layers in interface cultures. Molds grown in interface cultures showed mycelia elongated into the hydrocarbon layer without elongating into the aqueous layer. In this case, some molds excreted colored substances into the hydrocarbon layer, that is, Aspergillus terreus produced red pigment and Monascus purpureus produced yellow pigment.

It is very interesting whether molds growing under relatively anaerobic conditions assimilate hydrocarbons via oxygenation pathway[4] or via dehydrogenation pathway[5]. In general, bacteria and yeast growing aerobically were believed to oxidize hydrocarbons by oxygenation system[6,7]. However, no report has been published about the assimilation mechanism of hydrocarbons by molds, especially concerning the mechanism of the

Table 2. Growth of molds on the "hydrocarbon mixture 1" under the three cultural conditions.

Organism	Cultural condition: shaking		surface		interface	
	Cultivation time (days)					
	10	30	10	30	10	30
Aspergillus flavus OUT 5176	++	++	+++	+++	++	+++
A. fumigatus OUT 5008	++	++	+	+++	++	++
A. kawachii OUT 5017	+	+	-	+	+	+
A. niger OUT 5250	+	+++	+	+++	+	+++
A. oryzae OUT 5039	++	++	+++	+++	++	+++
A. oryzae var magnasporus OUT 5045	+	++	+	+++	+	+
A. terreus IFO 6123	++	++	+	+++	+	+
A. usamii IFO 4388	+	+	+	+++	-	+
A. usami mut. shiro-usamii IFO 6082	-	+	-	+	-	-
Penicillium chrysogenum OUT 2028	+	+	-	-	-	-
P. citrinum OUT 2125	++	+++	+	+++	+	++
P. expansum OUT 2038			+++	+++	++	++
P. notatum OUT 2050	++	+++	+++	+++	+	+
Fusarium moniliforme IFO 6349	++	++	+++	+++	+++	+++
F. oxysporium IFO 5942	+	+	+++	+++	++	+++
F. solani IFO 5890	-	-	-	-	-	-
Cladosporium resinae OUT 4077	++	++	+++	+++	+++	+++
C. resinae OUT 4295	+	+++	+++	+++	++	++
Monascus purpureus OUT 2013	-	-	-	+	-	+
Mucor javanicus OUT 1054	+	+	+	+	-	+
Neurospora crassa	+	+	-	-	-	-
Rhizopus tonkinensis	-	-	-	-	-	-
MH 201 (isolated from soil)	++	+++	+++	+++	+++	+++

Cultivation methods were shown in the text.
Symbols: +++=abundant growth, ++=moderate growth, +=slight, visible growth, and-=no visible growth.

anaerobic utilization of hydrocarbons. In this chapter we did not deal with the problem, but observed the evolution of a certain gas at the interface of aqueous layer and mold mat.

We also studied on the ability of some molds to grow on various hydrocarbons including aliphatic and aromatic ones. As seen in Table 3, Aspergillus flavus, Aspergillus niger, Penicillium notatum, and Cladosporium resinae 4077 were able to assimilate aliphatic hydrocarbons with carbon atoms more than 10, but could not utilize aromatic ones and n-alkanes of shorter chain-length. These organisms could also utilize higher aliphatic alcohols and acids, such as cetyl alcohol and palmitic and stearic acids. There are many reports regarding the mold growth on some hydrocarbon mixtures, such as Fusarium on diesel fuel[8] and Cladosporium on jet fuel[9]. In our study, several molds could grow on kerosene and light oil. These included Aspergillus flavus, Aspergillus niger, Aspergillus oryzae, Penicillium notatum and Cladosporium resinae. These were also able to assimilate wax and paraffin (m.p. 42-44°C), as shown in Table 3, but utilized pure n-alkanes or n-alkane mixture more readily than crude hydrocarbon mixtures mentioned above.

Table 3. Growth of molds on various carbon sources.

Carbon source	*Aspergillus flavus*	*A. niger*	*Penicillium notatum*	*Cladosporium resinae*
n-Hexane	-	-	-	-
n-Heptane	-	-	-	-
n-Hexadecane	+++	+++	+++	+++
n-Octadecane	+++	+++	+++	+++
Cyclohexane	-	-	-	-
Benzene	-	-	-	-
Toluene	-	-	-	-
Xylene	-	-	-	-
Ethylbenzene	-	-	-	-
Naphthalene	-	-	-	-
Phenanthlene	-	-	-	-
Paraffin (m.p. 42-44°C)	++	+	+++	++
Wax	++	+	+	++
Light oil	++	-	++	++
Kerosene	+	+	+	+
Hydrocarbon mixture 1	+++	+++	+++	+++
Cetyl alcohol	++	+	+	+
Palmitic acid	++	+	+	++

Stearic acid	++	++	++	++

Each carbon source was added at the concentration of 2.0 v/v or w/v %.

Molds were cultivated statically (surface culture) for 2 weeks at 30°C.

Symbols; +++=abundant growth, ++=moderate growth, +=slight, visible growth, and -=no visible growth.

4. Summary

The capability of several kinds of molds to grow on hydrocarbons was investigated under three different cultural conditions; shaking culture, surface culture and interface culture. Some strains of Aspergillus, Penicillium, Fusarium and Cladosporium could grow under these three conditions using a hydrocarbon mixture (a mixture of n-undecane, n-dodecane, n-tridecane and n-tetradecane) as sole carbon source. Among these conditions, surface culture was considered to be most suitable for the growth of molds. All these organisms could assimilate n-hexadecane and n-octadecane, but relatively few could utilize light oil, kerosene, and/or paraffin (m.p. 42-44°C). Aspergillus flavus, Aspergillus niger, Penicillium notatum, and Cladosporium resinae, which could utilize n-alkanes of medium chain-length as carbon sources, could not assimilate n-alkanes of shorter chain-length (n-hexane and n-heptane) and aromatic hydrocarbons so far tested.

5. References

1). C.E. ZoBell: Adv. Enzymol., 10, 443 (Interscience Publishers, Inc., New York) (1950).

2). J.B. Davis: Petroleum Microbiology (Elsevier Publishing Co., Amsterdam) (1967).

3). A.J. Markovetz, J. Cazin, Jr. and J.E. Allen: Appl. Microbiol., 16, 487 (1968).

4). H.S. Mason: Adv. Enzymol., 19, 79 (Interscience Publishers, Inc., New York) (1957).

5). E. Azoulay and J.C. Senez: Compt. Rend. Acad. Sci., 247, 1251 (1958).

6). R.K. Gholson, J.N. Baptist and M.J. Coon: Biochemistry, 2, 1155 (1963).

7). M. Kusunose, J. Matsumoto, K. Ichihara, E. Kusunose and J. Nozaka: J. Biochem., 61, 665 (1967).

8). R.S. Flippin, C. Smith and M.N. Mickelson: Appl. Microbiol., 12, 93 (1964).

9). M. Inoue: Kei-Kinzoku, 16, 147 (1966).

General conclusion

The present study has been carried out to produce useful materials, such as some important vitamins and yeast cell mass, from petroleum hydrocarbons by fermentation technique.

At first, the author investigated the vitamin B_6 production by various microorganisms and screened a yeast, Candida albicans, as a potential vitamin B_6-producer. This yeast produced about 1 mg per liter of medium of vitamin B_6 on a hydrocarbon-salt medium supplemented with corn steep liquor and a non-ionic detergent.

The production of cytochrome c was next investigated using several kinds of yeasts. Candida lipolytica produced about 10 mg per liter of medium of cytochrome c on "hydrocarbon mixture 2" (rich in n-undecane). The relationship among the cell growth, the cytochrome c content and the respiratory activity of Candida albicans was also studied using n-hexadecane as a sole carbon source. The results showed that the close interrelationship existed between the growth rate and the cytochrome c productivity of this yeast. The growth rate of Candida albicans was enhanced by the addition of a detergent, Tween 85, and the results obtained suggested that the emulsification of hydrocarbon was one of the limiting

factor in the hydrocarbon fermentation. Another important factor was found out to be the maintenance of pH of the culture broth.

Third, the studies on the production and characterization of carotenoids produced by Mycobacterium smegmatis were carried out using several hydrocarbon substrates. The carotenoid production of this bacterium was largest on n-hexadecane and stimulated by addition of a non-ionic detergent and some amino acids, such as serine, histidine and glutamic acid. The carotenoids produced mainly consisted of 4-keto-γ-carotene and its derivatives. A water-soluble pigment produced by this bacterium on a hydrocarbon medium was also investigated. This pigment was found out to be coproporphyrin III from its spectral characteristics.

Fourth, we investigated the substrate specificity of several kinds of yeasts in hydrocarbon fermentation. Many yeasts grew well on n-alkanes of medium chain-length(C_{10-20}), but they could not almost assimilate higher fatty acids or alcohols. When Candida albicans was cultured on pure n-alkanes, n-decane, n-undecane and n-dodecane were the best substrates, n-tridecane, n-tetradecane and n-pentadecane were inferior, and n-hexane and n-octane were not utilized. This substrate specificity of Candida albicans was also observed when several

hydrocarbon mixtures (mixtures of $\underline{n}$-C_{10-14}) were used as a sole carbon source.

Publication list

1). Studies on the formation of vitamins and their functions in hydrocarbon fermentation III. Production of vitamin B_6 by several kinds of yeast in hydrocarbon media.
A. Tanaka and S. Fukui : J. Ferment. Technol., 45, 611 (1967)

2). Studies on the formation of vitamins and their functions in hydrocarbon fermentation IV. Production of vitamin B_6 by Candida albicans in hydrocarbon media.
A. Tanaka, N. Ohishi and S. Fukui : J. Ferment. Technol., 45, 617 (1967)

3). Studies on the utilization of hydrocarbons by micro-organisms I. Production of cytochrome c by Candida albicans in hydrocarbon fermentation. Part I. Production of yeast cells from hydrocarbons.
A. Tanaka, H. Maki and S. Fukui : J. Ferment. Technol., 45, 1156 (1967)

4). Studies on the utilization of hydrocarbons by micro-organisms II. Production of cytochrome c by Candida albicans in hydrocarbon fermentation. Part II. Production of cytochrome c by Candida albicans.
A. Tanaka, H. Maki and S. Fukui : J. Ferment. Technol., 45, 1163 (1967)

5). Studies on the utilization of hydrocarbons by microorganisms III. Substrate specificity of yeast in hydrocarbon fermentation.
A. Tanaka and S. Fukui : J. Ferment. Technol., 46, 214 (1968)

6). Studies on the utilization of hydrocarbons by microorganisms IV. Growth of molds on hydrocarbons.
A. Tanaka, S. Shimizu and S. Fukui : J. Ferment. Technol., 46, 461 (1968)

7). Studies on the formation of vitamins and their functions in hydrocarbon fermentation V. Production of carotenoids by Mycobacterium smegmatis in hydrocarbon media. Part I. Studies on the cultural conditions.
A. Tanaka, T. Nagasaki, M. Inagawa and S. Fukui : J. Ferment. Technol., 46, 468 (1968)

8). Studies on the formation of vitamins and their functions in hydrocarbon fermentation VI. Production of carotenoids by Mycobacterium smegmatis in hydrocarbon media. Part II. Isolation and characterization of several carotenoids produced by Mycobacterium smegmatis IFO 3080.
A. Tanaka, T. Nagasaki and S. Fukui : J. Ferment. Technol., 46, 477 (1968)

9). Studies on the utilization of hydrocarbons by micro-

organisms V. Accumulation of porphyrin in culture filtrate of Mycobacterium smegmatis during hydrocarbon assimilation.

A. Tanaka, T. Nagasaki and S. Fukui : J. Ferment. Technol. (in press)